SYMPOSIUM ON NUTRITION

THE PHYSIOLOGICAL ROLE OF CERTAIN VITAMINS AND TRACE ELEMENTS

Edited by

ROGER M. HERRIOTT

Foreword by

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FOREWORD

The Symposium on Certain Vitamins and Trace Elements, of which this volume is a record, realized to an unusual degree the objectives of those who planned the program Over a period of several decades pioneering studies have been replaced by an increasing number of techniques, both chemical and biological These have resulted in a more adequate concept of the chemical natures of the individual nutrients discussed here and of the anatomical responses to deprivation or inadequacy of each specific nutrient Paralleling these advances in knowledge of the nature and distribution of the vitamins discussed, clinical studies have added greatly to our understanding of the symptomatology, pathology, and geographical distribution of the diseases resulting from deficiencies of specific nutrients In common with the development of other departments of science, the contributions to the field of nutrition are to be found in a wide variety of technical journals and publications In these can be found descriptions of methods of study, speculation, criticism and interpretation of experiments

The contributors to this symposium are investigators of distinction They are exceptionally qualified to express their views on what is established fact and what is still debatable They have given us their matured interpretations of the findings in the fields in which they are specialists Discussions by such able investigators add immeasurably to our progress toward a complete understanding of our nutritional problems

The writer of this foreword is highly honored by the expressions of courtesy and esteem shown him by the willingness of the notable contributors to give their time and energy to the preparation of manuscripts and to attendance and participation in the program That they did this to make possible a symposium held as a tribute to the writer affords him the greatest satisfaction

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ELMER VERNER McCOLLUM

Emeritus Professor of Biochemistry
The Johns Hopkins University

Elmer Verner McCollum was born on a farm in western Kansas near Fort Scott, on March 3, 1879 His education began in a typical one room schoolhouse located about two miles from his home and continued there until he was seventeen

In 1896 Dr McCollum's parents sold their farm and moved to Lawrence to make it possible for the children to attend high school At first there was some doubt about Elmer's qualifying for admission to high school and it looked as though a written examination would be necessary to prove his fitness An understanding principal quizzed the candidate on his general knowledge and reading activities On the basis of his performance he was admitted without examination for a month s trial If, at the end of this period, the report was satisfactory nothing more would be said about entrance requirements In the words of the uneasy student himself " a great load fell from me with these words Apparently the report of my first month's performance was satisfactory to him for nothing further was said about it

In 1900 Dr McCollum entered the University of Kansas From this institution he received the B A degree in 1903 and an M A in 1904 Interest in organic chemistry, especially the synthesis of pyrimidines, led to the selection of Yale University as the best place for graduate study Here he obtained a scholarship which paid his tuition for the first year To supplement his income he taught a course in chemistry at the Y M C A Ultimately he secured enough tutoring to assure adequate funds for a more comfortable living

In 1906 Dr McCollum received his Ph D in organic chemistry under Professor Treat B Johnson The same year he also received the coveted Loomis Award for excellence in chemistry

Unable to obtain the kind of position he wanted he remained at Yale during 1906 07 and studied in the laboratory of Dr Lafayette

B Mendel In addition to the work in the laboratory he attended the lectures of Mendel, Underhill, and Chittenden

In the summer of 1907 Dr McCollum joined the staff of Professor E B Hart, then head of Agricultural Chemistry at the University of Wisconsin He participated with Hart and others in experiments suggested by Dr S M Babcock to determine the actual nutritive value of feeds by means of biological as well as chemical procedures In six years at Wisconsin Dr McCollum rose rapidly from the rank of instructor to that of full professor He established at the University of Wisconsin the first rat colony in America maintained for nutrition studies

A variety of significant developments in the field of nutrition have stemmed from fundamental research initiated by McCollum His investigations at the University of Wisconsin marked the beginning of study of chemical aspects of animal nutrition and led to major improvements in feeding farm animals In studies with small animals he demonstrated that nucleic acid, lecithin, cephalin, and phosphorized proteins can be synthesized by the body The next outstanding contribution was his discovery of the first of the fat soluble vitamins, vitamin A Two years later Dr McCollum reported an achievement of equal distinction in discovery of the animal s need for a water soluble nutrient, later called vitamin B At the same time he formulated the first adequate working hypothesis concerning what, in chemical terms, constitutes an adequate diet He was also first to devise a method for the biological analysis of a food stuff

In 1917 Dr McCollum was made head of the Department of Biochemistry of the newly established School of Hygiene and Public Health at the Johns Hopkins University Here he continued his investigations on the biological analyses of familiar foods and feed stuffs At the instigation of the late Dr John Howland, then head of Pediatrics at the Johns Hopkins Hospital, he turned his attention to the study of the etiology of rickets In collaboration with Dr Edwards A Park and other members of the Department of Pediatrics he succeeded in producing rickets in the rat and revealed relationships of dietary calcium and phosphorus to normal skeletal devel-

ment This research also led to the discovery of vitamin D and to development of a biological test for assay for anti ricketic products Further studies on vitamins resulted in discovery of the first chemical method for determining vitamin B_1 and in investigation of vitamin E in relation to dietary fat and muscular dystrophy

Experiments on the role of inorganic elements in nutrition resulted in a series of reports by McCollum and his associates on the nutritional indispensability of magnesium, sodium, and potassium Work in this field was continued with pioneer investigations on the physiological role of the trace elements, aluminum, manganese, fluorine, zinc, nickel, cobalt, and boron Dr McCollum has recently reentered this field in his capacity as a member of the advisory board of the McCollum Pratt Institute, of the Johns Hopkins University, established in his honor for study of the role of micronutrients in plant, animal, and human nutrition

Dr McCollum was made emeritus professor of Biochemistry in 1946 Almost as active in retirement as in the busy years as professor, Dr McCollum is continuing his investigations in a laboratory at the Homewood campus of the Johns Hopkins University He is a busy consultant His writing activities in many fields continue undiminished Through his own work, and through the work and teachings of his students, Dr McCollum has exerted, and will continue to exert, a profound influence upon the sciences of biochemistry and nutrition

Dr McCollum is a member of many distinguished national and foreign scientific organizations and a recipient of numerous awards and medals He has been awarded an honorary Sc D by the University of Cincinnati, honorary LL D by the University of Manitoba and the Johns Hopkins University He is a member of the National Academy of Sciences, American Philosophical Society, The Harvey Society, Royal Academy of Medicine of Belgium, Swedish Academy of Sciences, Fellow of the Royal Academy of Arts and Sciences, and many other similar organizations He is also a member of Sigma Xi, Phi Beta Kappa and Alpha Chi Sigma For twenty years Dr McCollum was an editor of the Journal of Biological Chemistry



DR. ELMER VERNER McCOLLUM

SYMPOSIUM ON NUTRITION

THE PHYSIOLOGICAL ROLE OF CERTAIN VITAMINS AND TRACE ELEMENTS

VITAMIN A IN THE NUTRITION OF THE NEWBORN

PAUL H PHILLIPS
University of Wisconsin

THE WORD NEWBORN is used in this presentation in its broad sense I shall really use it to indicate the nutrition of early life It includes the effects of vitamin A upon conception, gestation, intrauterine development, placental transfer, fetal liver storage, contribution of milks and lactation In this presentation no effort has been made upon the part of the author to make an exhaustive search of the literature on the subject However, an attempt has been made to use pertinent references to the main body of the literature There seems to be a paucity of data on the subject especially in relation to certain species, including man

A basis for the appreciation of this problem began with the classical experiments of Hart and coworkers (35) in the period between 1907 and 1911 These workers fed rations composed of single grains and their by products in an experiment with cattle which was among the forerunners of modern nutrition In this work it was shown among other things that the animals fed the wheat ration failed in reproduction The basis for the elucidation of this problem can be rightly credited to the early pioneer work of E V McCollum (56)

In order to understand properly the physiological effects of a marginal or submarginal dietary deficiency of vitamin A, it is helpful to visualize its histopathology Avitaminosis A primarily results in an alternating cycle of stimulation and exhaustion, with final complete exhaustion of the cell which at this point undergoes congestive atrophy and necrosis The epithelial lining and covering cells of the body in a vitamin A deficiency undergo keratinizing metaplasia, while other structures such as the intracellular bodies may be affected as shown by Popper (68) Wolbach and Bessey (88) described the sequence of the epithelial effect as atrophy of the epithelium

concerned, a reparative proliferation of the basal cells, and growth *and differentiation of the new products into a stratified keratinizing* epithelium' Sutton and coworkers (75, 76) among others, have stressed the fact that nerve degeneration occurs in a vitamin A deficiency, while Madsen *et al* (52) report cystic pituitaries in cattle While it is true that the changes in the nervous system may be secondary pressure phenomena, the author does not consider the present evidence complete and conclusive In the young calf, hepatic cells may undergo nuclear condensation, degeneration, and necrosis accompanied by lymphocytic infiltration (9, 62, 79) From these pathological effects it is to be expected that many diverse symptoms may appear and many functions stimulated or depressed depending upon the severity of the vitamin A deficiency

The information on the effect of vitamin A on conception rate is quite limited, although the existing evidence indicates that a marginal dietary vitamin A reduces the conception rate Avitaminosis A causes a cessation of spermatogenesis in both laboratory as well as farm animals (21, 26, 39, 41, 52, 53, 55, 75, 88) and hence prevents con ception Spermatogenic failure follows cellular damage to the germinal epithelium of the testis and to the development of cystic pituitaries (cattle) According to Erb and coworkers (21) these effects in pre pubertal bulls resulted in seriously impaired fertility and reproductive capacity since sexual drive and spermatogenesis were reduced Sterility and cessation of oestrus have been noted (rats and cattle), as well as prolongation of gestation, difficult parturition, and retained placental membranes (cattle) It is obvious from these results that vitamin A is needed for the production of sperm and ova capable of fertilization

The type of placental membrane appears to regulate the amount of vitamin A which is transferred through the maternal blood supply to that of the developing fetus (Table 1) In many species there is sufficient transfer of vitamin A to permit limited stores of this vitamin in the liver of the young at birth In others, the multi membranous type placenta prevents the transfer of excess vitamin A Lund and Kimble (49) administered large amounts of vitamin A (20 000 330 000 I U) to pregnant women and observed the vitamin

TABLE 1

COMPARATIVE BLOOD PLASMA VITAMIN A CONCENTRATION OF DIFFERENT SPECIES AT BIRTH WITH THE NORMAL OF THE SPECIES

Species	Plasma vitamin A μ/100 ml	
	Avg at birth	Normal for species
Pigs	< 5	20 30
Lambs	6	25 35
Calves	< 6	20 30
Human	10	40 60

A content of the maternal and fetal blood The data indicated that the average normal blood plasma level in the adult nonpregnant female was 96 I U per cent while that of the fetal blood average only 49 I U per cent, a reduction of roughly 50 per cent They believed that the plasma A of the newly born infant was independent of the maternal plasma vitamin A and the mother s intake, and concluded that vitamin A was poorly transmitted from mother to fetus That the problem is not an acute one in normal women fed typical American diets is shown by the work of Byrn and Eastman (6) These investigators studied the comparative blood plasma vitamin A levels of maternal and fetal bloods and arrived at a conclusion similar to that of Lund *et al* They pointed out that the fetal plasma vitamin A values cannot be raised by increases in the maternal diet

The cow for example has several placental membranes which effectively preclude the wholesale portage of vitamin A through the structure They form an effective filter or barrier unless the maternal blood vitamin A is elevated by extraordinary ingestion levels

However, in the case of cattle, massive dietary doses of vitamin A result in an increased storage in the livers of the young This has been amply demonstrated with calves by Spielman *et al* (73), Dann (9), Eaton *et al* (17), Fontaine *et al* (24), and Wise *et al* (85), among others Thomas *et al* (78) and Benham (1) came to the conclusion that increased placental transfer was attained by increasing the maternal dietary intake The effects of the increased ingestion

caused an increase in blood plasma, liver and colostral vitamin A concentrations It would seem that under ordinary dietary conditions in most species there is normally only a limited storage of vitamin A in the liver of the newborn animal

There is ample evidence to show that the ingestion level of caro tene or vitamin A influences the amounts of vitamin A stored in the maternal liver, and that it also influences the relative transfer of the vitamin either via the placenta or the milk The experimental data support this statement, and also the wide experience of producers of farm livestock Osborne and Mendel (61), as early as 1918, demonstrated that the liver was the principal organ of storage of vitamin A, a fact that has subsequently been amply confirmed The liver content of vitamin A under most conditions is proportional to the intake The storage apparently takes place against considerable odds although Clayton (8) points out that certain agents which affect the liver itself, do not affect the vitamin A concentration per se

While there may be considerable variations in the vitamin A stored in the fetal liver during pregnancy, and granting that species differ ences may cause still greater variations, it remains a fact that the stores of vitamin A at birth are relatively low Hart and Guilbert (38) state that the livers of newborn animals tend to be low in vitamin A, irrespective of the diet of the dam during gestation This statement is in line with the author's observations and experi ence as well as the work of others

Wolff (89) studied the liver vitamin A content of full term and premature infants and despite considerable variations found that the vitamin A content of both was low, i e, only one third that which was found in adults dying by accident These observations have been confirmed in their general features by Toverude and Ender (80), Ellison and Moore (20), Neuweiler (59), With (86), and others Dann (11) reported low reserves in newborn rats and rabbits Eekelen and Wolff (18) observed that young kitten and newborn puppy livers were low in vitamin A Debré and Busson (13) likewise reported low fetal liver storage in newborn puppies and newborn infants Braun and Carle (5) studied the liver vitamin A of aborted fetuses from cows on a brucella experiment and observed

low liver concentrations Braun and Carle's data are exceedingly interesting in that they reflect placental transfer in proportion to the dietary intakes of their dams although supplementary vitamin A failed to greatly enhance the liver vitamin A A portion of their data is summarized as follows

No of cows	Dietary treatment	Liver vit A I U /g (avg)	Fetal vit A I U /g (avg)
7	Low A	95 4	1 7
5	Low A + 800 000 I U /week (shark oil)	335 0	14 1
4	Poor pasture (low A)	220 2	6 6
5	Poor pasture + shark oil 800 000 I U /week	391 1	10 7

They concluded that the vitamin A content of fetal liver, 'although low, was in direct relationship to the mother s diet" In a study by Eaton and associates (17), cows were given a million units of vitamin A daily for 30 days or a total of 30,000,000 units each This produced an average storage of 2,438 μg in the livers of 10 day old offspring as compared to 140 μg for livers of calves from control cows which did not receive supplemental vitamin A The difference between these values is indeed striking and represents a 17 fold increase When the vitamin A requirements of the calf are considered, how ever, the differences fade into insignificance, since the daily require-ment for vitamin A in the young calf is in excess of 3,000 I U per 100 pounds of body weight

Spielman *et al* (73) were able to obtain storage values of 97,177 I U per liver for calves from cows fed 30 million units daily as compared to 1,319 to 2,355 I U for calves from cows fed normal diets This amount (97,000 units) is about equal to that which a calf would normally obtain from colostrum during the first 24 hours of life The reader s attention is directed to the relatively little stored vitamin A that is available to the newborn calf Krauss and coworkers (44) have shown that the amount of carotene and vitamin A in the liver of the newborn calf is extremely low

Phillips and coworkers (63) in an attempt to relate nutrition to diarrhea of calves studied the blood plasma vitamin A concentrations

of the newborn calf at birth and for several days and weeks there after In 16 newborn calves the blood plasma vitamin A content was 4 μg per 100 ml in comparison to the normal 18 μg or more per 100 ml of blood These newly born unnursed calves were from well fed, well managed dairy cows maintained by the University of Wisconsin at Madison The authors concluded that calves are normally born deficient in vitamin A Further, they were able to present some evidence to show that calves from cows fed winter rations were lower in their vitamin A content than those dropped by cows on pasture Blood plasma concentrations were 4 and 8 μg /100 ml of blood, respectively, for the two groups with the latter range of 8 μg definitely in the borderline zone according to the work of Boyer *et al* (4) These data would support the concept that little placental vitamin A is transferred beyond that needed for the growth and development of a normal fetus

Lund and Kimble (49) compared the plasma vitamin A content of umbilical cord blood to the maternal venous blood and found the plasma vitamin A in the newborn infant to be approximately half that of the normal adult The plasma vitamin A values of the newborn infant were independent of the maternal plasma vitamin A regardless of the mother s intake of the vitamin They concluded that the fetus and the mother are never in equilibrium as regards vitamin A except by chance Their evidence suggested that there was little or no transmission of vitamin A through the placenta in either direction Gaehtgens (25) regarded the placenta as a partial barrier to vitamin A Lund and Kimble (49) suggested that the lipid content of the placenta limited the passage of vitamin A In 1946 Popjak (67) demonstrated that increased lipid concentration in the placenta retarded the movement of maternal nutrients to the fetus Williamson (83) fed high cholesterol diets (up to 8 per cent) to female rats and succeeded in increasing the placental lipids and thereby reduced the fetal liver and body stored vitamin A by 50 per cent From these data it appears that both the structure of the placental wall and its lipid content affect the vitamin A transfer through the organ With the exception of extremely high doses of vitamin A administered to dairy cows which were presumably fed

an ample ration of the vitamin or its precursors, the preponderance of the data indicate that the newborn animal has stored within its own reserves at most only a very small quantity of vitamin A It is important and in some cases imperative, therefore, that vitamin A be given in generous amounts to the newborn Sobell and coworkers (72) have shown that aqueous dispersions of vitamin A are better utilized by four times than oily dispersions when fed to mother rats as measured by liver storage of the vitamin in the young

Hentges *et al* (40) studied the relationship of blood plasma concentrations to liver vitamin A content, night blindness and cerebrospinal fluid pressure in the young pig These workers found that the blood plasma concentrations of vitamin A remained within the normal range until the liver stores are quite low or depleted The threshold for borderline plasma concentrations of the blood vitamin A were from 8 to 10 μg /100 ml The appearance of increased cerebrospinal fluid pressure occurred when the blood plasma level dropped to 7 μg /100 ml , and night blindness developed only when the concentrations were below 5 μg /100 ml

The principal avenue of transfer of vitamin A from the mother to the mammalian offspring is by means of the colostrum milk Macy *et al* (51) demonstrated that pooled human milk from women maintained on an average American diet would support growth and reproduction in the rat fed a vitamin A low diet Thatcher and Sure (77), on the other hand, reported a vitamin A deficiency death of an infant breast fed from a poorly nourished mother The vitamin A content of human and cows colostrum, according to Dann (12) is quite similar but human milk is richer in vitamin A than cows milk He reports that the feeding of cod liver oil during pregnancy in women did not increase the vitamin A content of the colostral milk The colostral milk contained 632 I U /100 ml (total biological activity) as against 346 I U /100 ml for normal milk He estimates that human colostrum has the biological vitamin A activity equivalent to three fifths of that of cows colostrum These data are valid for the conditions under which they were obtained, but they should not be used to draw the generalization that they are apropos to all conditions Such factors as the ampleness of liver stores, previous biologi

cal stress such as the number of lactations, frequency of pregnancy, disease or illnesses, age and vigor, may, one or all, affect the vitamin A content of milk and its contribution to the diet of the young

The enrichment of the diet with large quantities of vitamin A results in an increased secretion of vitamin A in the milk Hrubetz (43) and coworkers fed varying amounts of vitamin A from the sixth month of pregnancy One group was unsupplemented while the others were divided into 3 groups which received 50,000, 100,000, and 200,000 I U daily until lactation was terminated There were no deleterious effects observed from feeding these levels of vitamin A These levels represent intakes of 10, 20, and 40 times the vitamin A requirement for the human

EFFECT OF VITAMIN A ADMINISTRATION TO PREGNANT AND LACTATING WOMEN UPON VITAMIN A OF MILK

(Hrubetz *et al* (43))

Group	1	2	3	4
Period	Controls	50 000 I U	100 00 I U	200 000 I U
2 10 days	424	747	1037	1137
61+ days	281	344	885	718

In 1941 Deuel (14, 15) gave massive doses of vitamin A in shark liver oil to dairy cattle fed excellent rations and noted a marked increase in the vitamin A content of the milk The efficiency with which these large doses of vitamin A were absorbed and utilized was found to be in the order of 0 78 to 2 91 per cent for Guernsey cattle and 2 47 to 4 60 per cent for Holsteins when 700,000 I U were given daily

The importance of colostrum in the nutrition of the newborn calf has been emphasized by many workers, among them Liebscher (48), Smith *et al* (71), Drummond and coworkers (16), Lundquist and Phillips (50), Dann (10), and others Colostrum is nature s greatest single food for the mammalian newborn Parenthetically, it is one of the best foods produced on our farms even for the human Hansen and Phillips (30) found the high protein content of cows colostrum was composed largely of a globulin fraction (12 to 13 per cent) which was characterized electrophoretically as gamma globulin and that this globulin was absorbed apparently intact (29, 31) by the

calf during its first 24 hours of life Thereafter, no absorption could be detected by measurements on the blood of a proper test animal Later studies by Hansen, Potter and Phillips (34) revealed that the amino acid content of water soluble globulin of bovine colostrum was similar in many respects to human globulin but differed in that it was higher in proline and isoleucine This work clarifies and amply confirms the work of Howe (42) and Ehrlich (19) Howe observed that salt fractionating of blood serum produced proteins which he called euglobulin and pseudoglobulin Newborn calf blood does not contain these protein fractions but upon ingestion of colostrum these proteins appear in the blood of the nursling In 1922 Orcutt and Howe (60) demonstrated that the agglutinins of brucella abortis appear in the blood of the calf after and only after the ingestion of colostrum, thus demonstrating that immunity is transmitted from the dam to the offspring through the colostrum milk Erhlich, as early as 1892, in a classical experiment, immunized young mice against specific protein antigen Tests upon young mice demonstrated that young born of immune mothers had antibodies transmitted *in utero* as they were present at birth and before the young had a chance to nurse Further, mice born of nonimmunized mothers were not immunized but developed specific antibodies after nursing colostrum from immune mice There is little doubt that these researches clarify the importance of colostral immunization through direct transfer of certain specific proteins and that this attribute of colostrum milk is independent of the vitamin A content Infection in avitaminosis A is a secondary effect to the dysfunction of epithelial structures damaged by want of adequate vitamin A On the other hand, Stewart and McCallum (74) related certain types of infection to the vitamin A content of the colostrum, a fact supported by the work of Phillips and coworkers (63, 64) Dann in 1933 (10) estimated that the calf received 10 to 100 times as much vitamin A from colostrum as from an equal quantity of milk The work of Semb and coworkers (70) indicates that colostrum may carry 5 to 15 times more vitamin A than normal milk

Many factors affect the vitamin A content of colostrum Carotene or vitamin A ingested by the dam influences the vitamin A in the

milks As already pointed out the season makes a difference in the vitamin A content Dann (10) reported in 1933 that he observed wide variations in cattle of the same breed and eating identical feeds He found that heifers colostrum contained two times more vitamin A than colostrum from older cows Hansen, Phillips and Smith (33) fed young dairy cattle and noted a marked drop in colostral A with succeeding lactations (Table 2) Colostrum from first calf heifers

TABLE 2

EFFECT OF NUMBER OF LACTATIONS UPON VITAMIN A CONTENT OF COLOSTRUM (vitamin A—γ/100 ml)

Lactation	1	2	3
Number of cases	21	19	5
Average vitamin A	317	187	139

averaged 308 μg/100 ml compared to their second lactation colo strum average value of 139 μg/100 ml when they were fed similar rations Furthermore, some colostral milks were too low in vitamin A to furnish more than the borderline of requirements of vitamin A for the newborn calf Esh *et al* (22) fed lecithin with vitamin A to cows during the late stages of gestation and observed greater placental transfer as well as increased transfer to colostrum The amount of vitamin A in sows milk colostrum is less than in cows milk as shown by Bowland *et al* (3) Lambs, like calves, are born deficient or low in vitamin A, and the transfer of vitamin A takes place through the colostrum which is about six times richer in vitamin A than normal ewe milk according to Pope, Phillips, and Bohstedt (66) and Pierce (65) Pierce not only observed low blood plasma concentrations in lambs but found that the livers were also low Both the blood plasma concentrations and liver stores rose rapidly with the ingestion of colostrum

Growth rates in the young are impeded by lack of vitamin A The effect upon growth is to a large extent dependent upon the severity of the deficiency Wolbach and Bessey (87) have presented evidence which indicates a differential slowing of the growth process

in the developing and growing animal They postulate a sharp curtailment of skeletal growth while the central nervous system continues to grow in the avitaminosis A of early life, thus creating pressure and stress in the cerebral spinal cavity which in turn cause skeletal changes (88) which eventually cause nervous lesions as a result of mechanical injury in the vitamin A deficient animal Moore and Sykes (57) have measured the cerebral spinal fluid pressure and correlated a marked increase in pressure with a developing vitamin A deficiency They considered this to be a reliable and sensitive test for avitaminosis A It is unlikely that this type of change occurs in the very young animal because other organs and structures of the body become more rapidly affected and the animal is lost before the more slowly developing growth differential of the neuroskeletal systems has the opportunity to express itself

It has already been pointed out that dietary vitamin A or its precursors profoundly affects conception, spermatogenesis, gestation, placental transfer, fetal liver storage and its transfer from mother to young through colostrum and milk Each of these gross effects of vitamin A deficiency is an expression of the degree of avitaminosis The author s (62) experience agrees well with those of Hart and Guilbert (38) in that reproduction failure varies in character with the nature and the degree of deficiency (1) A severe vitamin A deficiency will interfere with estrus and spermatogenesis, hence a failure to breed (2) A less severe deficiency permits breeding but may result in infertility or resorption of young as well as in lactation failure (3) Still less severe deficiency permits breeding and con ception but does not allow normal *in utero* growth and development of the fetus Warkany and Schaffenberger (81) by feeding a submarginal amount of carotene to young female rats until breeding age and then placing them on a vitamin A deficient ration obtained conception in a sufficient number to determine the effect upon the *in utero* development Seven females out of 149 were reported to have carried their young to term and approximately half of the latter were stillborn Congenital deformities were obtained in the offspring which showed defects of the skeleton and eye This work was later confirmed by Wilson and Barch (84) by the use of

Warkany s approach and with the further precaution of 8 controls which were handled as the experimental animals but were given 150 μg β carotene weekly On the other hand, Cannon in an earlier report (7) was unable to demonstrate congenital abnormalities in the rat with the use of purified diets Hale (28) obtained piglets with microphthalmos, macrophthalmos and anophthalmos The work of Ross *et al* (69) and Nell and Phillips (58) suggests that other dietary factors may have been involved in the deficiencies produced by Warkany, Wilson, or Hale Further evidence in support of this suggestion is found in the work by Folley *et al* (23), Henderson *et al* (39), Maruyama and Phillips (54), and Bowland *et al* (2) These workers were unable to obtain normal reproduction with purified diets which contained supposedly adequate vitamin A The investi gations of Hart, McCollum, Steenbock, and Humphrey (35) demon strated with cattle that a well balanced ration from the wheat plant alone resulted in birth of premature, very weak or dead calves The reproductive failure was most prevalent in cows fed the all wheat ration followed closely by those animals fed the all oat diet, while animals fed the all corn (carotenoid containing) ration produced normal calves By 1920 these workers (36) were able to demonstrate that the all wheat ration, if supplemented with calcium and butter fat, would support reproduction with some degree of satisfaction Because of the developing knowledge of vitamin A in nutrition, Hart, Steenbock, Humphrey and Hulce (37) reinterpreted the results of their previous experiment, and they were led to conclude that in light of our modern views of nutrition it is perfectly clear that the wheat ration was deficient in vitamin A and calcium ' Hart and Guilbert (38) of the California Experiment Station record the occurrence of a vitamin A deficiency in range cattle grazing for long periods on drought stricken grasses The cows on these pastures gave birth to dead or weak calves with or without eye lesions Retained placentae stimulating contagious abortion were associated with production of the weak and dead calves The calves which lived exhibited diarrhea similar in character to white scours The eye lesions resembled infectious keratitis

It seems necessary to mention vitamin A requirements for the

growing young We agree with Guilbert and Hart (26) that the vitamin A requirement of animals is directly related to body weight since it is the author s opinion that all cells of the body except fat cells require the vitamin A for their intimate metabolism

In reviewing this subject it is well to keep in mind the diversity of avitaminosis effect upon the animal body and its functions and realize that requirements will vary dependent upon the criteria of evaluation used The growth rate of young, vaginal smears, liver storage, cerebral spinal fluid pressure, nyctalopia, blood plasma vitamin A concentrations among others have been most widely used A complicating factor involved in assessing the requirements is the fact that an animal is exposed to two sources of vitamin A (1) dietary vitamin A potency and (2) liver storage of vitamin A or its precursors Liver storage A is difficult to control or eliminate as a variable because of the ease of the vitamins release from, or the tenacity with which it is held by hepatic structures The young, newborn animal makes a particularly valuable experimental animal in this regard because of the low vitamin A concentration in the liver at birth

Guilbert, Miller and Hughes (27) made use of nyctalopia supple mented with the antimony trichloride test on liver extract as a criterion for the requirements of cattle, sheep and swine They came to the conclusion that these species require from 6 to 8 μg per kg of body weight of vitamin A and 25 to 30 μg of carotene per kg of body weight per day as the physiological minimum Boyer *et al* (4) showed that 6 μg per kg of body weight permitted deficiency symptoms to develop while it required 3 times this level of 18 μg per kg body weight to provide adequate vitamin A (as measured by blood plasma vitamin A concentration, health, and vigor) in young calves These workers found that the carotene requirement was roughly 4 times the vitamin A requirement, a ratio in line with that of Guilbert, Miller and Hughes (27) These requirement figures are in close agreement with those of Lewis and Wilson (47) for growing calves and with those of Lewis and Haig (46) for the requirements of the infant and those of Lewis *et al* (45) for the young rat These workers report the near minimum requirement

of the calf as 32 U S P units or 8 μg per kg of body weight per day and the minimum requirement of the infant and rat were the same order of magnitude, that is 5 to 9 μg per kg of body weight per day The minimum requirement for the young growing animal must be at least doubled to avoid dipping into the deficient state in times of stress, and it should be tripled to obtain excellent growth, vigor, proper health and well being These studies would then

TABLE 3

THE RELATIONSHIP OF VITAMIN A SUPPLEMENTS TO SURVIVAL AND BLOOD PLASMA VITAMIN A CONCENTRATION

	10 000 I U Vitamin A conc /day		25 000 I U Vitamin A conc /day		
	+ N A or *nicotinamide*	Vitamin A *only*	Vitamin A *only*	Vitamin A + *niacin*	Vitamin A + *biotin*
No of calves	6	3	12	16	7
			Plasma vitamin A per 100 cc		
Age					
0	5 0	3 0	4 2	4 5	5 0
24	6 0	4 5	8 6	9 5	9 0
150 250	11 0	——	11 0	11 5	11 0
% Survival	50 0	33 3	75 0	75 0	86 0

place the minimum requirement at 3 0 to 4 μg per pound of body weight with 8 to 10 μg per pound body weight per day as an adequate dietary requirement

There is some evidence to indicate that the requirement for vitamin A by the newborn is greater during the first few days of life than at later stages of growth and development (Table 3) Hansen, Phillips and Rupel showed (32) that the needs of the newborn calf were in excess of 10,000 I U per 100 pounds of body weight per day when severe diarrhea and survival were used as the criteria of the minimum requirement This is in contrast to a daily requirement of 3,000 to 5,000 I U necessary for the same size calf at 3 to 8 weeks of age

SUMMARY

From this discussion it is readily appreciated that vitamin A or its precursor is an absolutely essential dietary factor required by all newborn animals It appears to be one of the most important nutrients needed in the early life of the newborn, both *in utero* and during the nursing stage It is required by the adult male and female as a fundamental essential for the development of the reproductive cells Submarginal vitamin A brings about a variety of abnormalities in the development of the young Resorption of the new life *in utero*, fetal death and expulsion, stillborn young, skeletal abnormalities, or ophthalmic defects occur in the order of decreasing depletion Nutritive inadequacy in the diet of the newly born results in a characteristic syndrome as follows excessive lachrymation (intermittent at first), respiratory congestion and infection, intermittent diarrhea becoming chronic and finally very severe, debility followed by pneumonia and death These symptoms appear most frequently in the order named although their appearance may be simultaneous in the rapidly advanced case These symptoms are the direct results of injury to epithelial cells and structures

The amount of vitamin A stored in the liver of the newborn is extremely small under ordinary conditions and hence provision for the maternal transfer of this vitamin is provided through the colostrum, a milk 10 to 100 times richer in vitamin A than normal milk

In summary then it would appear that young animals are born with a minimal storage of vitamin A in reserve Hence the dietary requirement for vitamin A of the newborn is high until the habilitary needs are met, thereafter 8 to 10 μg per pound of body weight will adequately meet the young animal s requirements The placental membranes act as a barrier to the *in utero* transfer of large quantities of vitamin A or carotene This barrier action can be modified by dietary means but in nature the principal means of transfer to the young mammal is via the colostrum and milk An early transfer of vitamin A by means of an easily digested and absorbed source of the vitamin is absolutely essential not only to quickly habilitate the cells of epithelial origin but to activate the intra cellular structures of

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Discussion

Dr Sobel This portion of the subject was so well reviewed that I don't think I have too much to add The problem of the transfer of vitamin A across body membranes that are impaired and thus produce a local or overall deficience concerns me both theoretically and experimentally, and, as a matter of fact, in many of my investigative relationships with the Pediatrics Department This problem of the proper nutrition of newborns after birth goes back more and more to that of understanding the feeding of the mother in order that the developing young get proper nutrition Our experiments seem to indicate that at least the question of the transport of fat soluble vitamins is not too well understood It is the function of the particle size and interfacial tension in the transfer of vitamin A across intestines into the blood stream The transfer from the blood and other transport systems across the placental barrier is a more difficult problem Our studies on enrichment of milk vitamin A indicate that serum levels are a significant factor, as one might predict

from theoretical considerations of membrane transfer The transfer of vitamin A from the diet (via the blood) to the milk is usually very poor when the 'A' is given in oil When we gave aqueous dispersions of 'A' we got considerable transfer of A' to milk However, when we produced corresponding blood levels by giving large doses of oily 'A" (compared to aqueous A'), we obtained corresponding transfer to milk Thus it seems that one of the factors involved in this membrane transfer is serum concentration Other factors, of course, have to do with the innate nature of the membranes, and the study of these membranes will probably clarify and may provide practical solutions for the problem presented by Dr Phillips so well As a matter of fact, as I explain again, the field he presented is quite new to me I haven t been as well up on the subject as I thought, and I learned a great deal

DR HICKMAN Do you have any comments on that, Dr Phillips?

DR PHILLIPS I'd just like to mention one difficulty in getting at the problem There are two sources of vitamin A—one is the liver and the other is the exogenous dietary source and there are always limitations to blood vitamin A studies If the liver is completely depleted then vitamin A blood plasma values accurately reflect the nutritive condition of the animal On the other hand, if there is some storage in the liver the blood plasma vitamin A doesn't reflect much because it is influenced by the release and storage of vitamin A by the liver structures and so we ve felt that we ve had to have a depleted animal for actual measurement Thus far from our studies with laboratory species and experimental animals, where we could study both liver stores and blood plasma vitamin A it appears that they are all low or deficient in vitamin A at birth regardless of the placental transfer of the placental structure and that we have to give a tremendous dose of vitamin A in order to influence the storage in the newborn We have come to the conclusion, as far as practical application to farm livestock is concerned, that the best thing to do is to feed the young rather than to try to transfer it through the mother basically because of the economic considerations

DR SOBEL May I add something to this? We studied the transfer of vitamin A to the milk in cows, following the intravenous injection of vitamin A, the same amount of aqueous A by mouth, and also, the same amount of vitamin A dissolved in oil I may add that intravenous injection in the cow seems to be far more easily carried out than giving it to them by mouth via stomach tubes The standard transfer was the amount found in blood after intravenous injection The same aqueous material given by mouth was 40 per cent of the intravenous value, and the same amount of vitamin A given in oil was 10 per cent of the intravenous value Now, in farm animals I think that intravenous injection of aqueous dispersions is a practical procedure that

can affect the transfer of vitamin A from the mother through the placenta into the growing embryo

DR HICKMAN Dr Quaife, do you have any comments to make?

DR QUAIFE I guess the only comment that I could make is that I m struck with the similarity of the activity of vitamin A in the animal body as compared to that of vitamin E I can t help thinking that you could almost extend the story of vitamin A in the cow to all mammalian species I d like to congratulate Dr Phillips for his excellent presentation

DR DEUEL There is an interesting problem in relation to feeding these excessive doses of vitamin A to the cow which as yet has not been answered although it has been confirmed When you give 700 000 or a million and a half units of vitamin A to a cow, you find within a very short period a period of one to two weeks, that the carotene rapidly diminishes to a minimum value and that the decrease is maintained over the period of the feeding and for three or four weeks after this We have repeatedly noted that change It is so obvious that the butter which we prepared from our milks was practically white This is a phenomenon which is not limited to the cow because we were able to reproduce it in chickens in which the yolks of eggs became practically colorless after dosage with high levels of vitamin A Beta carotene is probably not the main coloring agent in the yolk as it is in the milk I bring this up at this time because I m rather uncertain whether Dr Moore will consider it and I am wondering whether Dr Phillips has any suggestions I might say that our own theory for this phenomenon is that, in order to get rid of this excessively high amount of vitamin A the body builds up a rather strong enzyme system which attaches beta ionone structures other than vitamin A This may be a possible explanation We have repeatedly confirmed the finding and there must be some physiological explanation Do you have any experience, Dr Phillips, on that phase?

DR PHILLIPS None, except that we have confirmed your work—namely, that the feeding of high vitamin A does reduce the carotene Why it takes place I don t know I wondered in connection with the problem if perhaps there might be a differential absorption of carotene before it gets into the circulating fluids of the body That s mere speculation and we ve nothing to add further than what your suggestion implied

DR HICKMAN I would like to advance some information on that subject It is one we worked on a good deal some years ago Apparently what happens is that vitamin A catalyses oxidation in the rumen or intestinal tract of the animal and promotes destruction of some or all of the carotinoids One can duplicate the effect with a simple laboratory experiment A dilute solution of carotene in olive oil is poured into four test tubes The first tube is left

without further addition a few milligrams of tocopherol are added to the second a milligram of vitamin A is placed in the third and both E and A in the fourth Let the tubes stand on the bench for a week or two The second test tube full bleaches in a day the first in a week while the third and fourth stay colored indefinitely Vitam n A alone has rapidly bleached the carotene protected with E it fails to do so If excess vitamin A is adminis tered to a cow or chicken the milk and egg yolk become bleached if vitamin E or a synthetic fat soluble antioxidant is given simultaneously the vitamin A causes no bleaching The bleaching or not bleaching appears to be conditioned by the state of oxidation of the intestinal tract and I don t think we have to postulate any mutual balance or special biological mechanism The fact is that the cow has never had an opportunity in the millions of years of its develop ment to consume substantial portions of shark s liver and it has no protective mechanism against large quantities of free vitamin A As I watch these very interesting reports and compilations of blood and milk levels I cannot help but think the problem is one of transportation—how to get the nutrients from the food through the gut of the mother to the blood stream and hence through the placenta to the child regulated of course by storage depots along the way A question I would like to ask Dr Phillips is whether he feels that the serious obstruction imposed by the placenta to the passage of the fat soluble vitamins is a regrettable nuisance or has been placed there purposefully by nature so that in the former event nature has had to devise other ways of getting the fat soluble vitamins to the offspring for instance by way of colostrum milk?

Dr Phillips I m inclined to feel that it is not accident

Dr Hickman I thought not

Dr Phillips I think each of these structures in the body has a physi ological function and perhaps in this case it is a protection mechanism of the placental membrane and only highly water soluble materials can go across unimpaired The placental membrane effectively stops considerable transfer

Dr Hickman Would you say then that the placental membrane has been des gned to do a perfect job under optimum conditions and it only fails when

Dr Phillips Well that s what our data indicate

Dr De I would like to know Dr Phillips s opinion about the role of vitamin A in the production of vernix caseosa in the human newborn

Dr Phillips I have no opinion I m sorry

Dr Wald I wonder whether this matter of the placental barrier isn t a negative one rather than a positive one that is I rather think that the intestine also would be a very effective barrier to the passage of vitamin A

or any of the carotenoids I wonder if one isn't simply dealing here with the fact that bile salts, which are needed to bring the carotenoids through the intestinal barrier, are not present here to carry them through the pla cental barrier My other thought concerns the interesting interchange between you, Dr Hickman, and Dr Deuel concerning the depletion of carotene when one feeds a lot of vitamin A I think that your suggestion that this is due to peroxidation is a very interesting one, but I should think that it would be wise to check the elimination of carotene in the feces in order to check the alternative suggestion of Dr Phillips that it is not being fully absorbed It seems to me very likely that one has a limited mechanism for carrying carotenoids and in the presence of very large amounts of vitamin A, the carotene, which always is absorbed with great difficulty, perhaps doesn't get through or gets through only very slightly The thing that bothers me a little about the peroxidation idea is that I wonder how much oxygen is in the gut and whether you have any information on peroxidations there It seems to me that the gut is rather anaerobic

DR HICKMAN You can as a matter of fact change the picture in the gut from aerobic to anaerobic just by juggling the fat soluble and water soluble antioxidants We published rather extensively on that score some years ago, but apparently our work escaped the attention of the dairy chemists I hate to pose as an expert whenever this subject arises, but I feel a missionary duty to air my views What is the complete balance sheet accounting for the fate of a dose of vitamin A? Unfortunately we cannot do a complete autopsy or even a partial biopsy every few hours after ingestion, but we can make a fairly reliable imaginary accounting Our measurements suggest that about 24 hours after ingestion 25 per cent can be accounted for in increased blood liver and fat content plus fecal recovery If 100 mg of vitamin E is ingested at the same time, the computed recovery is 90 per cent, *both* blood and feces showing phenomenally higher values If oxidized cod liver oil is given instead of the vitamin E, the recovery sinks to zero or even becomes negative There is thus plenty of oxygen available in the intestinal tract if the conditions are right From day to day and season to season the oxidative reductive status of the gastro intestinal tract must swing over wide limits, and I imagine that each condition, *if not too prolonged*, serves a beneficial function Over the long pull, however, it is the reductive condition that is the important one, by all the evidence at our disposal

DR MASON With regard to placental transfer, I like to think that it represents physiologic conservation by nature to save those vitamin A stores for the newborn and flush them out in the colostrum, rather than that too much transferred across the placenta might harm the fetus Certainly it is a remarkable mechanism which, irrespective of marked variations in tissue layers intervening between maternal and fetal blood in various mammals,

mits vitamin transfer through the placenta in much the same manner Certainly the picture for vitamin A is almost exactly duplicated in the case of vitamin E and probably vitamin D also

DR HICKMAN That s another way of saying it s the transport problem

DR MASON It s the transport problem and I think that we might add further that colostrum has not received the attention it should have There are evidences that it contains important nutrients other than fat soluble vitamins which are much more concentrated there than in later milk If that is so should not some cognizance be taken of it in the formulation of the artificial formula for bottle fed infants such that the formula of the first few days be modeled more after the colostrum than the later milk

DR KRAMER I think this is a question of the importance of the barrier in the transport mechanism In the normal child where the intestine does not serve as such a barrier it is possible to raise to tremendous levels the vitamin A level in the blood When that occurs there develops a very definite syndrome of toxicity which has been described in the literature by Rodahl Rothman Caffey Tooney Gribetz and others

DR DARBY Along the same line perhaps or perhaps totally unrelated is the well established fact that during pregnancy there is a decrease in the blood level blood concentration of vitamin A as early in the pregnancy the level will have fallen slightly and then as one follows the same woman with repeated blood determinations the level will decrease until it has fallen perhaps 20 per cent or so of the original initial level by or during the third trimester Again one doesn t know or at least I don t know what the significance of this is but it has sometimes occurred to me that it may indeed be related to the considerations here of the undesirability if you will of pushing a great deal of vitamin A across to the fetus I d like to ask Dr Phillips if the same phenomenon occurs with bovine and other animals

DR PHILLIPS The same phenomenon does occur in other species as far as I know It often doesn t occur quite as early in pregnancy as it is indicated for women The question has concerned me for some time especially in dairy cattle where we re concerned with high levels of milk production The lactation period for cattle is terminated about two months before the next parturition During this period there is a regression of the mammary system and rebuilding for the next lactation period During the interval of the regression and reformation period colostrum is accumulating Again I don t know why we have concentrations of some of these vitamins in various structures in the body but it s my impression that the vitamin A passes into the mammary system and is there for a number of days before the milk is produced for release and it seems to me to concentrate in the gland itself I have seen no experimental work on this point but our observations indicate

VITAMIN A IN THE NORMAL INDIVIDUAL

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INTRODUCTION

IT IS NOW nearly 40 years since the classical studies of Dr McCollum and his colleague Davis (1913, 1914, 1915) led to the recognition of the existence of at least two accessory dietary factors, which they designated fat soluble A and water soluble B We are met together today to do honor to this great pioneer of vitamin research, and it is my privilege to contribute one of the papers on vitamin A On such an auspicious occasion, however, it seems only fitting that before inviting your interest in my own special subject I should ask you to look back, at least for a few moments towards the sources from which all our knowledge of vitamin A has been derived

It is often difficult, in tracing the source of a river, to decide which of two confluent branches should be described as the main stream, and which as a lesser tributary Some might argue that vitamin A was discovered by Hippocrates in 500 B C, or by an ancient Egyptian physician who treated night blindness with liver dipped in honey In the lapse of centuries, however, we might consider that this stream of knowledge had dried up in the desert, apart from a few trickles noticed by the explorer Livingstone and others We must look, therefore, to the higher level of pure research for the effective starting point of the main river of our present knowledge McCollum, like others, was trying to make rats subsist on diets which were made up of as pure food constituents as possible, and found, in effect, that the casein of milk was inadequate without supplements of both butter fat and water soluble substances Butter thus became the first

* I am grateful to the Robert Gould Research Foundation for the generous support which has enabled me to accept the invitation to take part in this conf

My thanks are also due to Dr I M Sh Mr R J Ward an Leitner for their cooperation in various experi ed or mentioned

source of vitamin A to be recognized In those early days we must not forget there was no antimony trichloride reaction available to reveal the presence of the vitamin in a few minutes, and it was truly a mysterious substance recognized only by its ability to cause growth in rats McCollum and Davis (1914) soon found, however, that this new factor had the very definite chemical property, unlike the fat containing it, of resisting saponification Some years later an inspiration came to Steenbock (1919) that the activity of the butter might be associated with its yellow color Here we had a tributary of major importance which brought in a wealth of knowledge of the relative vitamin A activities of all sorts of yellow and colorless vegetables, and which culminated eventually, after various twists and turns, in von Euler s demonstration of the vitamin A activity of pure carotene (1928) Our next flow of knowledge came from the realization that cod liver oil was a much richer source of vitamin A than butter, which doubtless gave Rosenheim and Drummond (1920) the brilliant idea that the well known purple reaction given by cod liver oil when treated with sulphuric acid must be due to the vitamin Takahashi and his colleagues (1925) in Japan, and Drummond *et al* (1925) in England made valiant attempts at the isolation of the vitamin from this source At this early stage the absorption band of vitamin A in the ultra violet region and the ill effects of greatly excessive doses were described by the Japanese workers

While these developments were being made from a chemical angle, the pathological effects of vitamin A deficiency were being recognized and described McCollum and Simmonds (1917) concluded that clinical xerophthalmia and the ocular symptoms of depleted rats were identical in character, and both due to deficiency of vitamin A Fridericia and Holm (1925) and others rediscovered the findings of the ancient Greeks and recognized the relation between night blindness and vitamin A deficiency Rickets, however, was not due to vitamin A deficiency, as proved by McCollum and his coworkers (1922) in a crucial paper which demonstrated that vitamin A was destroyed by oxidative treatment, as shown by Hopkins (1920), but that vitamin D was more resistant

Subsequent research on vitamin A has been prolific and fruitful The vitamin has been found in sources much richer than cod liver oil, and after years of work, both patient and inspired, has been isolated and eventually synthesized Various isomers of carotene and other provitamins, have been isolated from innumerable plant and animal tissues, and their conversion to vitamin A has been proved *in vivo* and *in vitro* Vitamin A, and its various modifications, have been studied in the human body and in common and rare species throughout the animal kingdom The estimation of vitamin A for commercial purposes has in itself become a responsible and highly specialized occupation and a crowded field of research A voluminous literature has appeared on the vitamin A requirements of farm animals The functions of vitamin A in the eye, which at present appear to have no parallel in other parts of the body, have been intensively examined both in America and Europe, and have allowed the vitamin to be studied as a component of an isolated enzyme system In the human the levels of vitamin A in the tissues and blood have been widely studied in health and disease

Out of all this wealth of knowledge it is my privilege to speak on the topic of 'vitamin A in the normal individual To keep my subject matter within reasonable limits of space and time I shall assume that the individual in which we are interested is human I shall commence, therefore, in Part 1 of my paper, with a review of the various investigations which have been made into the vitamin A levels in the liver and blood of normal human subjects, and into the effects of experimental deficiency To obtain information for application to the human, however, it is often necessary to resort to experiments with animals I shall attempt, therefore, to collect what evidence we have so far at our disposal on three problems which may well have important implications, and which at least have the fascination that we still seem a long way from learning the final answers Thus in Part 2 I shall attempt to discuss the factors which regulate the distribution of vitamin A in the body, in Part 3 the possible existence of new and concealed forms of vitamin A, and in Part 4 the value for the maintenance of health of a liberal, as opposed to a barely adequate intake of vitamin A As a necessary

preliminary I must ask your indulgence if at times I seem to place an undue emphasis on my own work, and that of my colleagues in Cambridge

PART 1 VITAMIN A IN THE NORMAL HUMAN

Vitamin A is normally present in much higher concentration in the liver than in any other tissue It was therefore only to be expected that in the years immediately following the development of the antimony trichloride method, when the Lovibond tintometer was the most accurate means of measurement available, estimations of vitamin A in human liver should have preceded its much more *difficult estimation in blood*

TABLE 1

LIVER VITAMIN A RESERVES

Country	Worker	Period	No of Cases	Vitamin A Mean	I U /g Median
Holland	Wolff	1929 32	78	160	110
Britain	Moore	1931 35	40	290	220
		1941 44	71	455	324
S Africa (natives)	Fox	1933	14	297	190
Finland	Skurnik	1936 38	19	(79)	(65)
China	Woo & Chu	1939	12	79	54
Norway	With & Ødegaard	1943	12	166	153
Sweden		1943-44	27	318	233
Scotland	Dzialoszynski & Tomaszewski	1947	11	586	638

Skurnik s values have been corrected for a presumed error in calibration

The first investigation on a large scale was made by Wolff (1929, 1932) in Holland, and several other studies have followed at intervals in various parts of the world (Moore, 1932, 1937, 1949, Fox, F W , 1933, Woo and Chu, 1939, Skurnik, 1944, With and Ødegaard, 1947, Dzialoszynski and Tomaszewski, 1947) The data given in Table 1 usually refer to groups of adult subjects who died by accident Most of the workers carried out many more examinations

on patients who had died from disease than on the victims of accidents, but the results obviously fall outside the scope of the present paper

It must be clearly understood that each of the medians given represents the central point of a very wide range In Britain we find values scattered between about 10 I U and 2,000 I U per g, although very low values have recently been uncommon The median value for Britain, which tended to increase during World War II, is somewhat higher than the medians for other countries, while the medians for Finland and China are outstandingly low In the Finnish work a Zeiss step photometer was used in place of the tintometer, and I have applied a correction to allow for a recognized error in the potency claimed for the German vitamin A concentrate which was used for purposes of standardization We cannot be confident, therefore, that the median for Finland was as low as it appears to be There seems no reason, however, to discredit the very low median found for China Although only a few specimens were examined from cases of accidental death, values below the European standards were also found in various diseases Values for a few Europeans who died in China, moreover, were considerably higher than for the native Chinese Thus the median for a group of 10 Europeans who died from accident or disease was 312 I U per g, which is nearly the same as the value we have found most recently in Britain

Liver Reserves of Vitamin A in Infants and Children

Numerous investigations of the vitamin A contents of the livers of both full term and premature infants have been made (Wolff, 1932, Toverud and Ender, 1935, 1, 2, Ellison and Moore, 1937, Woo and Chu, 1939, Skurnik, 1944) (see Table 2)

Early work by Dann (1932) indicated that vitamin A is transferred only in small amounts to the fetus in the rat and rabbit, and that the concentration in the fetus is not readily increased by raising the maternal intake of vitamin A or carotene The same general conclusion has since been reached for calves, lambs, piglets and many other animals It would perhaps be expected, therefore, that the human baby would possess only low reserves of vitamin A at birth

TABLE 2

VITAMIN A IN THE LIVERS OF INFANTS AND FETUSES

Country	Worker	No of Cases P	FT	Mean Vitamin A Premature	I U /g Full Term
Holland	Wolff 1932	18	24	41	44
Norway	Toverud & Ender 1935	47	50	65	39
England	Ellison & Moore 1937		11		27
China	Woo & Chu 1939	23	54	27	10
Finland	Skurnik * 1944	25	8	124	71

* Values corrected for error of calibration with Vogan

The extensive studies by Wolff, which were published at about the same time as Dann s work, appeared to support this view, although he found that in about one third of his cases the reserves reached values approaching the average concentration found for adults Ellison and Moore and Woo and Chu have also found decidedly lower vitamin A reserves in infants than in adults Skurnik (1944), Neuweiler (1936) and Marrack (1948), however, have found little difference between the concentration in the livers of *infants and adults*

An interesting tendency, which has been reported by Toverud and Ender, Woo and Chu Skurnik and also by Wendt (1936) but not by Wolff, is for the concentration of vitamin A in the livers of fetuses and premature infants to be higher than in infants born at full term

Only limited data appear to be available on the vitamin A reserves of growing children From Table 3 it will be seen that Ellison and Moore found that the reserves of babies who died in pre war days from all causes up to an age of 4 months were very low, but that between 4 and 8 months they were little different from the level found for children who had died by accident between the ages of 4 months and 14 years The median of 130 I U per g for this group, however, was only about half the median of 230 I U found for adults at that period For a small group of specimens from

on patients who had died from disease than on the victims of accidents, but the results obviously fall outside the scope of the present paper

It must be clearly understood that each of the medians given represents the central point of a very wide range In Britain we find values scattered between about 10 I U and 2,000 I U per g, although very low values have recently been uncommon The median value for Britain, which tended to increase during World War II is somewhat higher than the medians for other countries, while the medians for Finland and China are outstandingly low In the Finnish work a Zeiss step photometer was used in place of the tintometer, and I have applied a correction to allow for a recognized error in the potency claimed for the German vitamin A concentrate which was used for purposes of standardization We cannot be confident, therefore, that the median for Finland was as low as it appears to be There seems no reason, however, to discredit the very low median found for China Although only a few specimens were examined from cases of accidental death, values below the European standards were also found in various diseases Values for a few Europeans who died in China, moreover, were considerably higher than for the native Chinese Thus the median for a group of 10 Europeans who died from accident or disease was 312 I U per g, which is nearly the same as the value we have found most recently in Britain

Liver Reserves of Vitamin A in Infants and Children

Numerous investigations of the vitamin A contents of the livers of both full term and premature infants have been made (Wolff, 1932, Toverud and Ender, 1935, 1, 2, Ellison and Moore, 1937, Woo and Chu, 1939, Skurnik, 1944) (see Table 2)

Early work by Dann (1932) indicated that vitamin A is transferred only in small amounts to the fetus in the rat and rabbit, and that the concentration in the fetus is not readily increased by raising the maternal intake of vitamin A or carotene The same general conclu sion has since been reached for calves, lambs, piglets and many other animals It would perhaps be expected, therefore, that the human baby would possess only low reserves of v[illegible] A at birth

TABLE 2

VITAMIN A IN THE LIVERS OF INFANTS AND FETUSES

Country	Worker	No of Cases P	FT	Mean Vitamin A Premature	I U/g Full Term
Holland	Wolff 1932	18	24	41	44
Norway	Toverud & Ender 1935	47	50	65	39
England	Ellison & Moore 1937		11		27
China	Woo & Chu 1939	23	54	27	10
Finland	Skurnik * 1944	25	8	124	71

* Values corrected for error of calibration with Vogan

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children examined during the war (Moore, 1949) a much higher median of 550 I U was found It is encouraging to think that these results may reflect the great social progress which we have made in Britain in ensuring an adequate diet for our children

TABLE 3

VITAMIN A RESERVES IN THE LIVERS OF BRITISH INFANTS AND CHILDREN

Workers	Year	No of cases	Age Group	Cause of death	Median Vitamin A Reserve I U/g
Ellison & Moore	1935	11	0 4 wks	various	17
		21	5 wks 3 mos		14
		18	4 mos 8 mos		100
		12	4 mos 14 yrs	accident	130
Moore	1941 44	7	4 14 yrs		550

Vitamin A in the Blood Plasma

The concentration of vitamin A in the blood plasma is on an average about 300 times less than in the liver As already mentioned the Lovibond tintometer was only really suitable for examining liver, and reliable estimations upon blood only became possible with the adoption of a more delicate instrument A substantial advance in this direction was made when Dann and Evelyn (1938) demonstrated the value of the photoelectric absorptiometer for estimating vitamin A, and soon afterwards Kimble (1939) applied the absorptiometer for the examination of blood This she made possible by the develop ment of a simple and effective method of extraction, which has since been found satisfactory by many other workers Numerous investi gations have since been made in America by Abels, Gorham, Pack and Rhoads (1941), Murrill, Horton, Leibermann and Newburgh (1941), Yarbrough and Dann (1941), Brenner and Roberts (1943), Popper and Steigmann (1943) and others, in Britain by Yudkin (1941), Moore and Leitner (1949), Campbell and Tonks (1949) In South Africa Highman (1944) examined groups from the European, Indian and Bantu sections of the populations The mean values found by these groups of investigators are given in Table 4

TABLE 4

VITAMIN A AND CAROTENE CONTENTS OF THE BLOOD PLASMA OF HEALTHY ADULTS MIXED SEXES

Country	Observers	No of Subjects	Year	Total Carotenoids µg per 100 ml	Vitamin A I U per 100 ml
U S A	Kimble	64	*1939*	*176*	*109*
	Abels *et al*	124	1941	195	160
	Murrill *et al*	45	1941	206	104
	Yarbrough & Dann	16	1941	163	71
	Brenner & Roberts	6	1943	228	117
	Popper & Steigmann	27	1943	81	153
G B	Yudkin	23	1941	120	113
	Moore & Leitner	195	1944	92	121
	Campbell & Tonks	133	1947	80	108
S Africa *	Highman	40	1944	226	108

* Equal numbers of European males and females Means of 123 I U of vitamin A and 162 µg of carotene were found for 11 Indian males and 111 I U of 131 µg for 8 Bantu males

As found with liver the vitamin A content of the plasma varies widely between different healthy individuals, but the range covered is rather more restricted In subjects without obvious disease, resting values of less than 50 I U or more than 200 I U per 100 ml are at least uncommon For carotenoids the individual variation is perhaps somewhat wider and there are considerable differences between the mean values for different populations Thus in America the mean values with one exception ranged from 163 to 228 µg per 100 ml , as compared with 80 to 120 µg in Britain Even in the same country, moreover, considerable differences may be found between groups of the population living under different social conditions This point has been clearly demonstrated in surveys which Dr Sharman and myself have made in collaboration with Dr Z A Leitner, who has collected specimens of blood from his own private patients in London and from the inhabitants of a mental home at Claybury From Table 5 it will be seen that the mental patients gave values little different from those which we and other workers had found for the inhabitants of Britain, as typified by students,

manual workers, hospital out patients and patients in hospitals with diseases not expected to affect their carotenoid levels In contrast the private patients, mostly drawn from the wealthier classes, gave levels approaching those found by American investigators

TABLE 5

AVERAGE CAROTENOID AND VITAMIN A LEVELS IN THE BLOOD FROM PATIENTS AT CLAYBURY MENTAL HOSPITAL AS COMPARED WITH VALUES FOR PRIVATE PATIENTS SERVING AS CONTROLS

The specimens were received between March 8 and May 5 1951

		No of cases	Carotenoids µg /100 ml	Vitamin A I U /100 ml
Claybury patients	Male	20	50	123
	Female	25	62	116
Private patients	Male	35	116	171
	Female	24	139	138

The Sheffield Experiment on Vitamin Deficiency in Human Volunteers

During the war the adequacy of the vitamin A intake of the population of Britain gave rise to some anxiety at the Ministry of Food, where Sir Jack Drummond acted as Scientific Adviser A request was made to the Medical Research Council for more definite evidence than was then available on the daily requirement of vitamin A necessary for maintaining health Since carotene could be obtained at home in the form of carrots, whereas supplies of vitamin A depended largely upon American generosity, this point assumed special importance Information was also urgently required on the relative values of carotene and preformed vitamin A

Fortunately a group of volunteers willing to act as experimental subjects was already available at Sheffield, under the supervision of Dr Kenneth Mellanby This team had just completed trials on the propagation of scabies, and was placed at the disposal of the Vitamin A Sub Committee of the Medical Research Council Miss E M Hume, of the Lister Institute, acted as Secretary, and before long a team of about 20 scientists, including Medical specialists, had been recruited with the intention of examining the effects of deficiency from all possible angles

The Sub Committee had knowledge of the investigation organized in Germany by Wagner (1940) in which signs of deficiency had been developed within 6 months, and also of the work of Booher, Callison and Hewston (1939) in which defective dark adaptation had been observed after 16 to 124 days of depletion The proposed experiments were therefore expected to last from 6 to 8 months, and it was anticipated that there would be no difficulty in depleting sufficient volunteers to allow a study of the curative effects of graded doses of vitamin A and carotene in various forms Over sanguine hopes, however, were checked by the failure of Brenner and Roberts (1942) to produce signs of deficiency in three volunteers given a deficient diet 7½ months

Diet

To give the investigation every hope of success great care was taken to exclude all sources of more than traces of vitamin A and carotene from the diet A list of permitted foodstuffs reputed to contain only insignificant amounts was drawn up, and chemical tests were made in any doubtful cases Special supplies of dried skimmed milk, unvitaminized margarine, meat, bacon, sugar and jam were made available by the Ministry of Food Foods eaten in large quantities, such as bread and potatoes, were examined for carotenoids in laboratories at Cambridge or Liverpool, and as an additional precaution specimen meals were collected, dried and assayed for vitamin A by chemical means By this method both the Cambridge and Liverpool laboratories agreed that the average vitamin A intake of the volunteers not given supplements amounted to about 30 μg of carotene daily Finally specimens of the meals were collected, minced up without drying, and taken to Messrs Boots' laboratories at Nottingham for testing upon rats The animals did better than others given a standard basal diet deficient in vitamin A, in so far as they all survived over a period of 10 weeks They grew much less rapidly than animals given an adequate mixed diet, however, and had rough untidy coats At the end of the 10 weeks they were found to have no reserves of vitamin A in their livers, with the exception of a trace in one animal

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It may be agreed, therefore, that the volunteers received a diet which was as low as it could be made in vitamin A activity without either resorting to a completely artificial diet, or giving a diet deficient in nutrients other than vitamin A Sixteen volunteers, including two women, received the diet, at first without supplements, for periods ranging from 11½ to 25 months Five volunteers, including one woman, received the diet with prophylactic supplements of carotene for periods of 6¼ to 23 months Supplements of preformed vitamin A were given to two volunteers for periods of 18 and 22½ months Most of the volunteers were conscientious objectors to military service, and the mental attitude of self examination which they had revealed in relation to the war seemed to make them particularly reliable in adhering to the dietary restrictions which were imposed upon them

Evidence of Depletion

At fortnightly intervals all the volunteers submitted to the withdrawal of blood specimens by Professor H A Krebs, and specimens were posted to Oxford, Cambridge and Liverpool for estimations of carotene and of vitamin A by the antimony trichloride method Routine examinations for deficiency of dark adaptation were also made at regular intervals under the supervision of Professor Krebs For this purpose an adaptometer designed by Mr M Bartley, one of the volunteers, was mainly used, but a rod scotometer designed by Dr P C Livingston was also employed to locate faulty dark adaptation in different areas of the retina

The first effect of the diet, in those volunteers who were not given supplements of carotene, was a rapid fall in the level of carotenoids in the blood plasma At the start of the experiment the mean for the group was about 90 μg per 100 ml, which fell to about 20 μg within 8 weeks, and which remained in the region of 15 μg in the later stages of the experiment After 9 months of deprivation no α or β carotene could be detected by chromatographic analysis in a pooled specimen of blood from 12 of the volunteers As far as can be seen, therefore, the small residual levels of carotenoids which persisted throughout the experiment had no relation to the vitamin A story

The declines in the vitamin A level in the plasma, which ranged from 65 to about 120 I U per 100 ml in different individuals at the start of the experiment, were more gradual and showed greater variations between individuals One volunteer began with about 100 I U, and kept to the same level, apart from minor fluctuations, for about 2 years In eleven others vitamin A declined slowly, the average value of 88 I U soon after the start of the experiment only fell to about 70 I U in 9 months In the remaining four volunteers not given supplements, however, more rapid declines were observed From initial levels of about 100 I U the levels all fell to 40 I U, or under, within periods of 12 to 20 months

Curative Tests

The desired combination of a greatly reduced level of vitamin A in the plasma with defective dark adaptation was found in only three of the volunteers, who were therefore the only subjects available for curative tests One subject was given daily doses of 1,300 I U of preformed vitamin A, which gradually corrected dark adaptation and increased the level of vitamin A in the plasma towards its original level The two others were given carotene With 1,250 I U in oily solution the levels of carotene and vitamin A in the plasma were slightly increased, but doses of 2,500 I U were necessary to correct the faulty dark adaptation

Prophylactic Tests

The five volunteers in the control group who were given carotene received daily doses of 5,000 I U in various forms, including crystal line carotene in oily solution, sliced, canned or puréed carrots, canned spinach or dried cabbage The average levels of carotenoids in the plasma varied from 40 to 130 μg per 100 ml according to the source of carotene given, the average levels of vitamin A fell within the much narrower range of 81 to 112 I U The plasma of two volunteers who were dosed with 2,500 I U of preformed vitamin contained about 18 μg of carotenoids and 72 I U of vitamin A Dark adaptation did not become defective in any of the subjects dosed prophylactically with carotene or vitamin A

The services of the five controls who were dosed with carotene

were also used for investigating the efficiency of intestinal absorption, as measured by the difference between the amounts of carotene ingested and excreted in the feces The percentages lost by excretion were 26 with carotene in oily solution, 29 with carotene in margarine, 44 with canned homogenized carrots, 75 with canned sliced carrots, 57 and 59 with canned spinach in homogenized form or as purée, 59 with small doses of outer cabbage leaves and 73 with large doses

Requirements

On the basis of these observations, and allowing a wide safety margin, the daily requirement for preformed vitamin was estimated as 2,500 I U Alternatively the requirement could be met by 4,000 I U of β carotene in oily solution, 5,500 I U as homogenized carrots, 7,500 as cabbage or spinach and 12,000 I U as sliced carrots These suggestions, pieced together from the fragments of new evidence made available, have at least the virtue of agreeing well with previous estimates deduced mainly from a knowledge of the vitamin A and carotene contents of diets known to be satisfactory In particular they do not conflict with the U S A National Research Council s (1945) estimate of 5,000 I U of vitamin A and carotene combined, which is based on the assumption that approximately two thirds of the vitamin A value of the average diet is contributed by carotene

The outcome of the ambitious Sheffield experiment may be regarded with mixed feelings It was not found possible, as had been intended, to carry out curative tests with graded doses of vitamin A and carotene on an adequate number of subjects It must be fully realized, however, that the investigation had to be planned in the face of a serious and unavoidable handicap, since no investigator desiring to produce vitamin A deficiency within a reasonable time in experimental animals would choose to use adults known to possess substantial reserves Perhaps the most important conclusion to be drawn from the work is that the reserves known to be present in the human liver are used up gradually and economically during dietary deprivation, and are not dispersed with undue rapidity, as sometimes occurs in rats having very high reserves (Davies and Moore, 1935) If we take the median reserve in Britain as 324 I U per g (Moore,

1949) and the weight of the liver as about 1,500 g the typical reserve works out at about 500,000 I U On the evidence that most of the volunteers failed to show signs of deficiency within 500 days it would appear that the daily withdrawal of vitamin A from the liver is under 1,000 I U in the healthy adult The realization of unquestionable signs of deficiency in three out of sixteen subjects, therefore, may perhaps represent a greater scientific achievement, and a greater tribute to the patience and reliability of the volunteers, than might at first sight be appreciated

A detailed account of the investigation was compiled by Miss Hume and Dr Krebs, and has been published by the Medical Research Council (1949)

PART 2 FACTORS REGULATING THE DISTRIBUTION OF VITAMIN A, WITH PARTICULAR REFERENCE TO SEX

So far our story has been fairly straightforward The normal individual derives both carotene and vitamin A from his diet, and accumulates in the liver a substantial reserve of vitamin A, which is adequate for his prolonged survival on a deficient diet During such dietary deprivation the level of vitamin A in the plasma declines only slowly over a long period of months or years The position with carotene, however, is more hand to mouth, and the level in the plasma falls rapidly during the first few weeks of depletion

We must now face a more difficult problem in attempting to review such knowledge as is available on the factors which regulate the storage of vitamin A when excess is given, and its withdrawal from the liver for mobilization into the blood and other tissues during periods of dietary deficiency

It is well known that vitamin A is held in the blood mainly in the form of the free alcohol, and in the liver mainly in the form of esters When a heavy dose of preformed vitamin A is given, either in the free or esterified form, a temporary increase in the blood usually gives rise to a peak value within 4 hours after dosing, this increase is entirely made up by esterified forms of the vitamin After

24 hours the esters have usually completely disappeared from the plasma, and the total level of vitamin A in the blood is substantially the same as before dosing It is indeed remarkable to what extent the liver seems able to control the level of vitamin A in the blood plasma in the face of wide dietary variations Thus the work of Dr H W Josephs (1942) at Johns Hopkins University has shown that over a low range of concentrations there is adequate evidence that the level of vitamin A in the blood is related to its level in the liver, but beyond a certain point large additional doses of vitamin A may be absorbed by the liver with very little effect on the resting level in the plasma Since the resting level of vitamin A in the plasma is not simply related to its concentration in the liver we must look further for the methods by which the regulation is affected

Liver Operation, Nervous Stimulation

One of the earliest observations in this field was made by Drummond and McWalter (1935) in the course of attempts to prove the conversion of carotene to vitamin A in the liver When one lobe of the liver was removed from rats the concentration of vitamin A in the remaining lobes was found shortly afterwards to be much decreased

At about the same time Chevallier and his colleagues (Chevallier, Malméjac and Choron, 1935) reported that central stimulation of the pneumogastric nerve in dogs caused the level of vitamin A in the blood to be nearly doubled The same effect was observed after stimulating the splanchnic nerve Later the same workers (Malméjac, Chevallier and Choron, 1935) recalled that Bailly and Netter (1932) had observed that the suprarenal gland is rich in carotene, and designed experiments intended to decide whether nervous stimulation might exert its action on vitamin A through the medium of the suprarenals For this purpose pairs of dogs were anastomosed so that blood from the suprarenal vein of the one passed into the jugular vein of the other When the splanchnic nerve of the first dog was stimulated, the level of vitamin A in [illegible] n blood was not affected, but an increase was observed in [illegible] dog The [illegible] ect was observed even when the me[illegible] en remov[illegible] e suprarenal of the first dog

About 5 years later the problem was taken up independently by Young and Wald (1940) The observation by Drummond that removal of a lobe of the liver caused a reduced concentration of vitamin A in other lobes was confirmed in the rabbit In addition it was noticed that the level of vitamin A was increased as much as 5 to 7 5 times Vitamin A was also found to be increased in the blood by electric stimulation of the splanchnic nerve or by injections of adrenaline, but not by stimulation of the cervical sympathetics Young and Wald remarked that the mechanism responsible for the mobilization of vitamin A from the liver appeared to be the same as that responsible for the mobilization of sugar and certain blood proteins, and several other workers have also been attracted by this idea

Independent support for the claim that adrenaline mobilizes vitamin A into the blood was reported by Thiele and Guzinski (1940) who found that the level invariably rose after injections had been made into human patients The average magnitude of the increase, however, was only 25 per cent Hillman (1949) has recently reported that injections of epinephrine caused increases of vitamin A in the blood of 42 per cent of a group of normal subjects, but since in the remainder the levels were reduced, the means for the whole group before and after injection were not significantly changed In rabbits and rats Goodwin and Wilson (1949) have completely failed to confirm that adrenaline has any effect on the level of vitamin A in the blood

Alcohol

Conflicting evidence has also been obtained on the influence of ethyl alcohol in mobilizing vitamin A Clausen, Baum, McCoord, Rydeen and Breese (1940) found that the oral administration of alcohol to dogs caused the level in the blood plasma to be increased by from 50 to 300 per cent Later the same workers (1941) observed similar effects in humans In contrast Yudkin (1941) could detect no change in the level of vitamin A in human volunteers, even though there was evidence of improved dark adaptation In the Sheffield experiment heavy doses of alcohol failed to influence either the level

of vitamin A in the plasma or the efficiency of dark adaptation The result was equally negative in extra volunteers who were not restricted to the deficient diet

Sex

Evidence from several directions is available to prove that the distribution of vitamin A in the body is influenced by sex An early report by Ender (1934) stated the livers of cows contained more vitamin A than those of steers, but it is not certain whether the animals received the same diet, and were completely comparable in other ways In the human, Kimble (1939) found a slightly higher average vitamin A in the plasma for men than for women Most other workers have confirmed this finding, and it also appears that the relationship is reversed for carotene The figures already given in Table 5, for example, are in accordance with general experience No satisfactory comparison between the liver reserves of men and women has been possible, at any rate, in Britain, for the reason that many less specimens appear to be available from women who have died by accident than from men In regard to rats most workers are agreed that under the same conditions of nutrition the liver reserves of females tend to be higher than those of males (Kimble, 1939, Brenner, Brooks and Roberts, 1942, Callison and Knowles, 1945, Esh and Sutton, 1948, Booth, 1950) In the experiments of Lemley, Brown, Bird and Emmett (1947), however, no difference was observed between the stores accumulated by males and females Our own experience has been that the superiority of the female over the male tends to disappear as the dose is raised (Moore, Sharman and Ward, 1951)

Evidence from a different angle has been obtained in interesting experiments by Chapman, Gluck, Common and Maw (1949) who have examined the effect of injections of sex hormones into immature pullets Injections of oestradiol combined with smaller amounts of testosterone increased the level of vitamin A in the blood to 3 or 4 times the value found before treatment In normal six weeks old rabbits, however, Williamson (1947) found that oestradiol had little effect on the level of vitamin A in the plasma

The Influence of Sex in Maintaining the Normal Distribution of Vitamin A at Low Levels of Dosing

We may now turn to a rather complicated story, which shows that under carefully chosen experimental conditions sex may have a dramatic influence in determining the distribution of vitamin A between the liver and kidneys

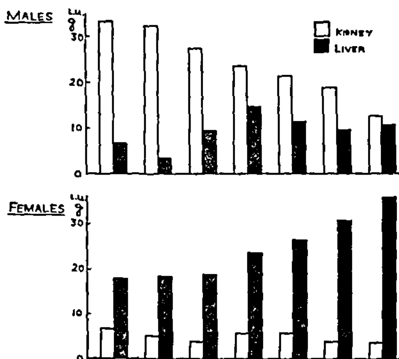


FIG 1 *Individual vitamin A concentrations in kidneys and livers for group given daily doses of 40 I U*

It has long been known that the main vitamin A reserves of the body are usually located in the liver Twenty years of research failed to reveal any important exceptions to this rule, but eventually Johnson and Baumann (1947) found that when rats were given relatively small doses of vitamin A or carotene, of the order of 35 I U daily, they accumulated much higher concentrations of vitamin A in their kidneys than in their liver

With various colleagues (Eden and Moore, 1950, Moore and

Sharman, 1950, Moore, Sharman and Ward, 1951) I have tried to follow up this important clue, and we have obtained further evidence on two points In the first place it appears that kidney is much more inclined to predominate over the liver in males than in females

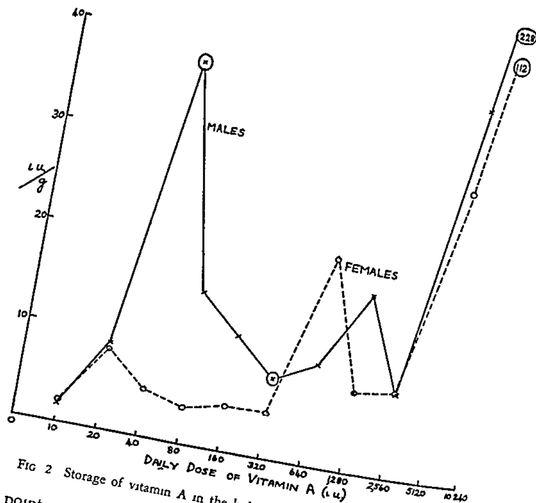


FIG 2 Storage of vitamin A in the kidneys of male and female rats

This point may be readily appreciated from Fig 1, which shows that in male rats receiving a daily dose of 40 I U the kidneys were richer in vitamin A than the livers, whereas in females the usual predominance of the liver was maintained Secondly we have been forced to the surprising conclusion that even in the male rat the accumulation of vitamin A in the kidneys is not increased, as might

be expected, but is substantially reduced by raising the intake of vitamin A beyond a certain level

This second point is illustrated in Fig 2, which shows the mean concentrations of vitamin A observed in the kidneys of groups of male and female rats at graded levels of dosing It will be seen that the concentration *in the male kidneys at first increased when the dose* was raised, then sharply decreased, and only rose again when very high levels of dosing were reached In females the maximum at low levels of dosing was much less pronounced

In Table 6 the relation between the kidney and liver in males is shown at two selected levels of doses, 40 and 320 I U By increasing the dose 8 times the average concentration of vitamin A in the liver was increased about 40 times, but in the kidneys was reduced to about one sixth of its level at the lower dose In these experiments the average growth rates in the two groups were virtually identical

TABLE 6

AVERAGE VITAMIN A VALUES IN GROUPS OF 5 MALE RATS EACH OF WHICH RECEIVED A BASAL DIET DEFICIENT IN VITAMIN A WITH SUPPLEMENTS AS INDICATED FOR 41 DAYS

Daily Dose I U	Mean growth in 41 days	Mean vitamin A I U/g Kidney	Liver
40	190	56	12
320	187	10	467

The Effect of Sex Hormones on the Concentration of Vitamin A in the Kidneys

Johnson and Baumann (1947) have found that vitamin A tends to accumulate in the kidney during growth It might be considered, therefore, that the greater concentrations of vitamin A in kidneys of male rats, as compared to those of females, could be ascribed merely to the well known fact that they grow much more rapidly It seemed of interest, therefore, to examine the influence of sex hormones on the distribution of the vitamin in castrated animals An investigation with male animals has already been completed (Moore,

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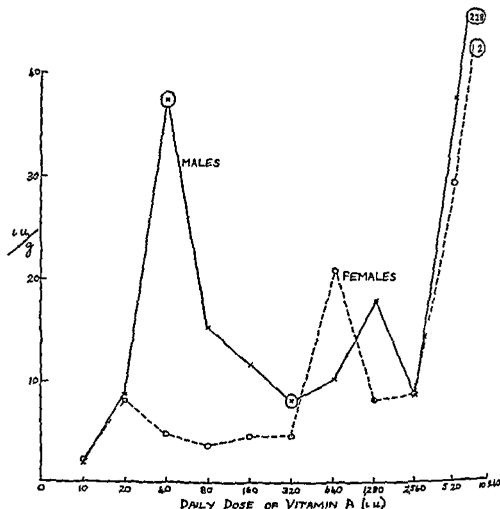


FIG 2 Storage of vitamin A in the kidneys of male and female rats

This point may be readily appreciated from Fig 1, which shows *that in male rats receiving a daily dose of 40 I U the kidneys were* richer in vitamin A than the livers, whereas in females the usual *predominance of the liver was maintained Secondly* we have been forced to the surprising conclusion that even in the male rat the accumulation of vitamin A in the kidneys is not increased, *as might*

be expected, but is substantially reduced by raising the intake of vitamin A beyond a certain level

This second point is illustrated in Fig 2, which shows the mean concentrations of vitamin A observed in the kidneys of groups of male and female rats at graded levels of dosing It will be seen that the concentration in the male kidneys at first increased when the dose was raised, then sharply decreased, and only rose again when very high levels of dosing were reached In females the maximum at low levels of dosing was much less pronounced

In Table 6 the relation between the kidney and liver in males is shown at two selected levels of doses, 40 and 320 I U By increasing the dose 8 times the average concentration of vitamin A in the liver was increased about 40 times, but in the kidneys was reduced to about one sixth of its level at the lower dose In these experiments the average growth rates in the two groups were virtually identical

TABLE 6

AVERAGE VITAMIN A VALUES IN GROUPS OF 5 MALE RATS EACH OF WHICH RECEIVED A BASAL DIET DEFICIENT IN VITAMIN A WITH SUPPLEMENTS AS INDICATED FOR 41 DAYS

Daily Dose I U	Mean growth in 41 days	Mean vitamin A I U /g Kidney	Mean vitamin A I U /g Liver
40	190	56	12
320	187	10	467

The Effect of Sex Hormones on the Concentration of Vitamin A in the Kidneys

Johnson and Baumann (1947) have found that vitamin A tends to accumulate in the kidney during growth It might be considered, therefore, that the greater concentrations of vitamin A in kidneys of male rats, as compared to those of females, could be ascribed merely to the well known fact that they grow much more rapidly It seemed of interest, therefore, to examine the influence of sex hormones on the distribution of the vitamin in castrated animals An investigation with male animals has already been completed (Moore,

Sharman and Ward, 1951), with results that are summarized in Table 7

TABLE 7

MEAN VITAMIN A VALUES IN KIDNEYS AND LIVER OF GROUPS OF 5 RATS GIVEN 40 I U OF VITAMIN A DAILY FOR 4 WEEKS

Group	Treatment	Mean wt increase (g)	Wt of seminal vesicles (g)	Kidneys		Liver	
				Total I U	I U/g	Total I U	I U/g
1	Entire animal	109	0 575	75 0	37 4	118	10 5
2	Castrated + testosterone	97	0 705	76 1	35 7	183	19 9
3	Castrated + testosterone (limited food intake)	40	0 575	25 7	18 7	250	37 6
4	Castrated + oestradiol	40	0 064	7 5	4 6	261	35 2
5	Castrated (no injections)	98	0 004	71 9	42 7	230	26 0

It will be seen that average concentrations of vitamin A in the kidneys were about the same in entire rats, castrated rats, and castrated rats with injections of testosterone Much lower concentrations, which might be taken as typical of females, were observed in the kidneys of castrated animals which were injected with oestrogen, and which grew much less rapidly than the animals in the three preceding groups In animals which were given testosterone, but which were pair fed with the oestrogen group, the concentrations of vitamin A were within the male range, but were lower than in animals treated with testosterone and given unlimited food

The above experiments, as far as they go, suggest that oestradiol stimulates the storage of vitamin A in the liver We must still attempt, however, to explain the paradoxical situation in which by increasing the dose we decrease the concentration in the kidney It seems necessary to assume that when the liver contains only a low concentration of vitamin A it is less efficient than usual in absorbing any further small supplies which may become available, and so takes second place to the kidney The tendency towards the abnormality may apparently be diminished by stimulating the liver with

oestrogen, or may be avoided by substantially raising the intake of the vitamin

In putting forward these rather curious results my confidence is strengthened by a friendly communication from Dr Baumann, who agrees that the kidneys of males tend to contain more vitamin A than those of females and that the amount of vitamin A in the kidney may not be decreased by raising the intake The same form of paradox has also been reported recently by Bodansky and Markardt (1951) These workers do not appear to have differentiated between the sexes, but have observed that an adrenal steroid, Reichstein s compound L, or 3 β acetoxy 17 α hydroxyallopregnan 20 one, has much the same effect as we have found for oestradiol in preventing the appearance of vitamin A in the kidneys We may perhaps recall the interesting point that the suprarenal capsules often contain very high concentrations of vitamin A (Davies and Moore, 1934)

Undue optimism that we are approaching a clear understanding of the action of hormones on vitamin A may be checked, however, by the results of our recent experiments on castrated female rats, in which the accumulation of vitamin A in the kidneys was found to be little affected by either oestradiol or testosterone

Other Factors

Mention must be made of two other agents which affect the metabolism of carotene and vitamin A, but which have not yet been clearly proved to influence distribution and mobilization Thyroxin has long been known to be concerned, whether directly or indirectly, in the conversion of carotene to vitamin A Thus Johnson and Baumann (1947) have found that the liver reserves of vitamin A accumulated by rats dosed with carotene are increased by the administration of thyroxin, and reduced by the thyroid inhibitors thiourea and thiouracil Vitamin E, moreover, has long been known to be necessary to allow the efficient storage of vitamin A in the liver (Moore, Martin and Rajagopal, 1939), to prevent the unduly rapid expenditure of the liver reserves (Davies and Moore, 1941), and to stabilize both carotene and vitamin A in the intestinal tract

(1948, 1) *supported this view by finding that rats first made deficient* on a basal diet of muscle peptone, starch, heated arachis oil, yeast, minerals and vitamin D gave at least a temporary growth response when their diet was changed to include casein and lard Incidentally he went on to extend his previous work by showing that livers taken from rats which had been cured by the diet containing lard were capable of causing temporary growth in rats still receiving the diet containing arachis oil As in the experiments with blood no vitamin A could be detected by chemical means

At this junction Randoin and Le Gallic (1948) reviewed the results of their early and recent experiments, and came to the con clusion that vitamin A and carotene act through the formation of a hormonal factor A, which is necessary to correct a faulty balance of nutrients Rats receiving a correctly balanced diet containing casein and lard were assumed to be capable of dispensing with vitamin A, which was essential when an incorrectly balanced diet containing muscle peptone and arachis oil was given Later Le Gallic (1949) found that his rats soon stopped growing when their diet contained lard in conjunction with muscle peptone, but that growth was resumed for 2 to 3 weeks when the peptone was replaced by heated casein In mice a similar response was obtained by replacing the peptone by either casein or dried egg albumin Subsequently *carefully dried cod muscle was found to give a greater* response when combined with lard than either casein or egg albumin (Le Gallic, 1950)

We must now cross the Atlantic and turn to the interesting work *of Kaunitz and Slanetz (1951, 1) These workers who apparently* were unaware of most of the French work, subjected lard to vacuum distillation and collected a volatile fraction containing 7 per cent of the original material Chemical and spectrophotometric examina tions indicated that not more than traces of vitamin A were present in this distillate, but unquestionably it showed biological activity when given to rats as a supplement to a diet deficient in vitamin A In a further communication the unknown factor was found to share with vitamin A the ability to protect rats against the harmful effects of rancid lard, it appears to be more resistant than vitamin A to

the destructive action of the products of rancidity (Kaunitz and Slanetz, 1950, 2) All the foregoing work would suggest that lard possesses biological activity which escapes detection by the usual chemical and physical methods It cannot be claimed to be a rich source of vitamin A activity, however, and if a new factor is involved we have no clue, at the moment of writing, as to its chemical nature

Metabolic Products of Vitamin A

The identification by Morton and Goodwin (1944) of the visual pigment retinene as vitamin A aldehyde provides the best evidence we have that vitamin A may undergo controlled oxidation in the tissues Unfortunately, for me, this observation has been made only in regard to the eye, and therefore falls in the field which will doubtless be covered by Dr Wald

Evidence of the metabolism, or possibly the destruction, of vitamin A in the general system, however, was reported about 10 years ago by Lepage and Pett (1941) whose investigations have hardly received the attention they deserve Healthy male human volunteers were first given massive doses of vitamin A, and four hours later extracts of the blood plasma were examined spectroscopically In many cases evidence was obtained not only of absorption maximum near the region expected for vitamin A, but also of another maximum at 275 mμ This second band was absent both from the sources of vitamin A which were administered and from extracts made from the blood of undosed volunteers Absorption at 275 mμ, however, was observed after treating fish liver oils rich in vitamin A with hydrogen peroxide It was concluded, therefore, that the absorption at 275 mμ seen in blood was due to an oxidation product closely related to the vitamin Absorption bands at the same position were also seen in extracts from the feces when they were collected at the appropriate interval after heavy doses of vitamin A had been given

With Mr R J Ward I have attempted to find similar evidence of the metabolism of vitamin A in the rat Vitamin A is lost more rapidly from the livers of rats deficient in vitamin E than from normal animals (Davies and Moore, 1941), and on the whole female rats

tend to store vitamin A more efficiently than males We thought, therefore, that we would stand the best chance of finding evidence of the metabolism of vitamin A if we used male rats which had been kept on a diet deficient in vitamin E, supplemented with small maintenance doses of vitamin A We also decided to pay most attention to the lungs, with the knowledge that large amounts of vitamin A may be accumulated in this site after heavy dosing, and in the hope that the vitamin might be rapidly metabolized in the profusion of epithelial tissues having ready access to oxygen

Our results are shown in Fig 3 The absorption spectrum found in an animal sacrificed 23 hours after dosing with about 100,000 I U of synthetic vitamin A indicates the presence of a high concentration of vitamin A in the lungs There already appears, however, to be slight differences in shape of the curve as compared with that given by the solution of synthetic vitamin A acetate used for dosing Thus the original maximum at 325 mμ has been supplemented by a somewhat higher maximum at 312 mμ, and slight inflections have appeared at about 295 and 280 mμ After 43 hours the main maximum is at 320 mμ, with a lower inflection at 310 mμ, while the inflections at 295 and 280 mμ have become much more pronounced After 160 hours and 184 hours the maxima at 280 mμ slightly exceed the maxima at 320 mμ, but at 280 hours the highest absorption is again at 320 mμ Finally we see that even in the undosed animal there is evidence of a slight inflection at 280 mμ

From these preliminary experiments it appears to us that we have evidence of the oxidation of vitamin A in the rat similar to that found by Lepage and Pett in human blood The substances which absorb at wavelengths shorter than 325 mμ appear to have only a transitory existence, unless we make the assumption that the substance in the undosed animal which absorbs at 280 mμ is an oxidation product of vitamin A This assumption, of course, would at present be quite unwarrantable *

* Note added March 26 1952 The extracts in the experiments with Mr Ward were made by digesting the lungs with alkali followed by extraction with ether In more recent experiments ether extracts made without the use of alkali have shown intense absorption bands at 296 mμ After saponification this band failed to appear, or was

Vitamin A Oxidized on Food

If evidence of at least the temporary presence of oxidation products of vitamin A in the tissues can be accepted our next question is whether these products retain any biological activity In this connection it is interesting to note that French workers have been responsible for the claim that unknown forms of vitamin A are produced when foods fortified with the vitamin are stored (Dubouloz, Marville and Chevallier, 1947, 1948, Dubouloz and Marville, 1950) Their story is that the foods after storage retain slight biological activity even when vitamin A can no longer be detected by careful chemical and spectroscopic examinations As with lard we have again no means of identifying by chemical or physical methods any new form of vitamin A which may have been produced

Vitamin A Acid

In all the foregoing work we may perhaps feel that we are being led on by a 'will of the wisp Substances having slight biological activity may in fact, as Dubouloz and his colleagues cautiously admit, contain amounts of vitamin too small for detection by the chemical means at present available The spectroscopic evidence of the metabolism of vitamin A in the blood or lungs, moreover, may reflect only the formation of inactive oxidation products arising from the uncontrolled destruction of the vitamin It is reassuring, therefore, to be able to turn to the consideration of vitamin A acid, a substance which differs widely from vitamin A, but which possesses at least some of its biological potency

It will be recalled that the synthesis of vitamin A acid was described about 5 years ago by Arens and Van Dorp (Arens and Van Dorp, 1946, 1, 2, Van Dorp and Arens, 1946, 1, 2) This substance has a general structure similar to that of vitamin A but with a terminal carboxyl group in place of the alcohol group In biological tests its potency was found by the Dutch workers to vary

present only as an inflection in the unsaponifiable fraction There is also evidence that the absorption of the unsaponifiable fraction may be influenced to some extent by the details of the manipulative procedure which was however, fairly well standardized in the experiments described in the text

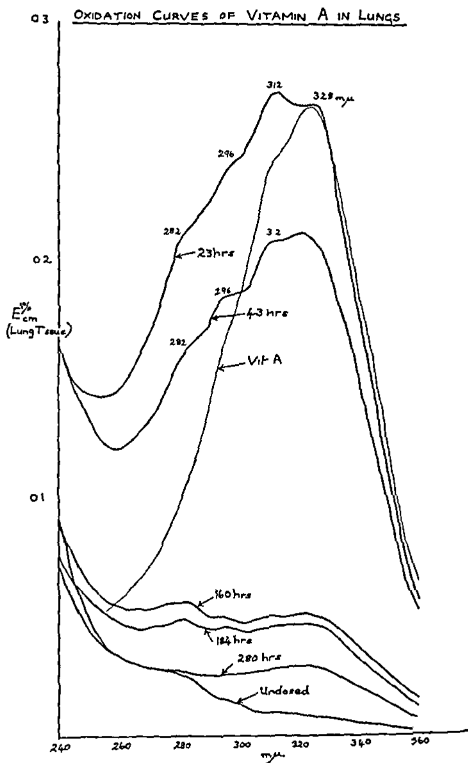

OXIDATION CURVES OF VITAMIN A IN LUNGS
0 3
0 2
0 1
$E^{1\%}_{cm}$
(Lung Tissue)
312
328 mμ
296
282
23 hrs
32
296
43 hrs
282
Vit A
160 hrs
184 hrs
280 hrs
Undosed
240
260
280
300
320
340
360
mμ

Fig 3

greatly according to the method of administration Thus growth promoting activity corresponding to 1 unit of the International Standard carotene could be supplied as 4 μg of the free acid dissolved in oil and given orally, as 0 6 μg of the sodium salt dispersed in water and given by subcutaneous injection or as only 0 3 μg of the sodium salt given orally The most surprising finding, however, was that the high biological activity of the acid was divorced from any ability to cause the storage of vitamin A in the liver

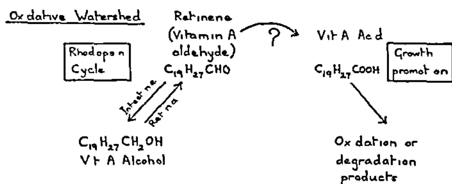


FIG 4

In my laboratory Dr I M Sharman (1949) has examined specimens of vitamin A acid kindly provided by the Dutch workers He has found no difficulty in confirming that the acid can restore growth in vitamin A deficient rats, although in oral dosing he found it considerably less active than carotene Even with doses of up to 10 mg of the acid no storage of vitamin A in the liver could be detected either spectrophotometrically or by the antimony trichloride method Neither could any evidence be found of the prominent absorption band at 349 mμ which is characteristic of unchanged vitamin A acid

Ball, Glover, Goodwin and Morton (1947) have reported that when retinene₁, or vitamin A aldehyde, is given to rats it is converted back to vitamin A in the tissues This certainly does not happen with vitamin A acid, and there would appear to be an oxidative watershed' between the two substances It is attractive to speculate that the different physiological roles of vitamin A may be

associated with different stages in its metabolism In fulfilling its function in the eye there may be a reversible cycle between the alcohol and aldehyde, but in promoting growth the vitamin may pass irreversibly to oxidation products, which may also be produced by the acid Perhaps I may be allowed to suggest to Dr Wald that it would be very interesting to examine the effect of vitamin A acid if any, in curing defective dark adaptation in animals deficient in vitamin A

Shrimps and Copepods

An account of hidden forms of vitamin A would not be complete without mention of the interesting reports from biological laboratories, but time cannot allow more than a brief reference

In Algeria, Grangaud and Massonet and their colleague Chechan have examined oils made from the shrimps, or prawns, *Aristeomorpha foliacea, Penaeidae* and *Aristeus antennatus* The oil collected from specimens caught during summer was red in color and contained astaxanthine, in various forms, but virtually no carotene or vitamin A In biological tests with rats it was highly active in curing xerophthalmia, but much less active in promoting growth Chromatographic and spectroscopic examinations indicated the possible presence of absorption maxima at 305 and 317 mμ Astaxanthin obtained from the oil without saponification was active against xerophthalmia, but astacene obtained after saponification was inactive (Grangaud and Massonet, 1948, 1949, Grangaud, Chechan and Massonet, 1950, Massonet, 1950, Grangaud and Massonet, 1950)

In contrast with these findings Kon and his colleagues (Kon and Thompson, 1949, Fisher, Kon and Thompson, 1951) have found preformed vitamin A in many species of shrimps, which have interested them as the food from which whales derive their substantial reserves of vitamin A It may be pointed out, however, that Kon found that vitamin A was concentrated in the exoskeleton, and particularly in the eyes, which appear to have been discarded by the French workers before extracting their oils

Very interesting observations have also been reported by Lane (1950) from Florida Oil was obtained from small shrimp like

celanoid copepods, *Temora turbinata* and *Centropagus typicus*, and was found to contain 1 6 mg of total carotenoids per g without any carotene When the oil was fed to the small fish *Limanda ferruginea*, however, it appeared to be much more effective than an amount of carotene corresponding to its carotenoid content in giving rise to storage of vitamin A in the liver Chromatographic separation gave fractions with absorption maxima at 430 and 310 mμ respectively When the fraction absorbing at 310 mμ was incubated with caecal brei from the *Limanda* the reaction for vitamin A in the antimony trichloride reaction was greatly increased

It seems possible, therefore, that we may have to deal not only with unknown factors which may arise from the metabolism of vitamin A, but also with unknown provitamins In this connection it is interesting to remember that Goodwin (1949) has observed that in the developing locust egg astaxanthin increases while caro tene falls If a change in the reverse direction could be established it would immensely simplify the whole story of vitamin A in the marine world

PART 4 BENEFITS DERIVED FROM A LIBERAL INTAKE OF VITAMIN A

It is well established that experimental animals may grow rapidly, and keep in apparently normal health, when given doses of vitamin A or carotene which are too small to allow the accumulation of measurable reserves of the vitamin in their livers It is also common knowledge that the livers of most animals which have been living under the conditions natural to them contain substantial accumu lations of the vitamin We have to face an important problem, there fore, in asking whether these substantial reserves, and the high intakes which give rise to them, are of any immediate benefit to health or are of value only as a provision for a future dietary emergency which may never arise

In Britain, where breast feeding is still the normal procedure for rearing infants, we have at least one clear instance of the vitamin A reserve acting as a safeguard against an immediate stress, since the

mother often secretes about 1,000 I U of vitamin A daily in her milk The special stresses on vitamin A metabolism caused by many diseases must also be remembered, but the topic falls outside the scope of the present communication We may concentrate, therefore, on seeing how far the question can be answered from the rather limited and fragmentary results which have so far been obtained upon experimental animals

The Optimum Vitamin A Supply for Long Life in Rats

Careful experiments on the optimum vitamin A intake for rats have been made by Sherman and Campbell (1945) and Sherman and Trupp (1949) These workers kept rats upon diets containing 3, 6, 12 and 24 I U per g of dry diet, which on the rough assumption of daily food intake of 10 g may be worked out as corresponding to total allowances of 30, 60, 120 and 240 I U daily Length of life, the span between sexual maturity and senility and reproductive records, were found to be greatest with the dose of 120 I U daily, *which gave slightly better results than either 60 or 240 I U* The vitamin A reserves found in the livers of other animals given the same allowances were 6, 71, 771 and 1,453 I U per g in males and 2, 165, 827 and 1,485 I U in females

The minimum dose necessary for slow growth in rats is generally considered to be about 2 I U In Sherman s experiments, therefore, a 15 fold increase above the minimum dosage allowed only traces of vitamin to appear in the liver For optimum health throughout life 60 times the minimum dose was needed, and it is most interesting that the reserves in the liver had now risen steeply to a value which would fall near the top of the range for wild rats, at least as found in Britain This is perhaps the best evidence which we have for the commonsense view that a reserve of about the normal size in the liver is conducive to normal health

Efficiency of Detoxication

The same problem has been approached from a less direct angle by Meunier, Ferrando, Jonanneteau and Thomas (1949, 1) in an interesting study of the effect of the vitamin A intake on the ability of rats to resist poisoning by sodium benzoate Rats given a basal

diet deficient in vitamin A with supplements of 2 5 μg of the vitamin daily grew fairly rapidly, but declined and died either when denied this supplement, or when given the supplement but with the further addition of sodium benzoate as 2 per cent of the diet Growth and survival could be effected, however, either by raising the allowance of vitamin A to 20 μg or by giving sufficient glycine to allow the benzoate to be detoxicated by conversion to hippuric acid It was concluded that vitamin A is directly concerned in the synthesis of glycine, and that when special calls on glycine are made the intake of vitamin A must be correspondingly increased Much the same conclusion had been reached, it may be remembered, by Manville (1937) who claimed that the failure of mucin formation in vitamin A deficiency was due to inadequate supplies of glycuranic acid, and that injuries resembling those caused by vitamin A deficiency could be produced in rabbits by giving them menthol

Similar experiments were made later with 1 per cent of bromo benzene as the toxic agent It was again found that the poison became harmless when the dose of vitamin A was raised to 20 μg The administration of cystine, with which bromobenzene is coupled during detoxication, did not however appear to be effective In experiments with two different types of poisons, therefore, rats given liberal doses of vitamin A were able to resist metabolic stresses which proved fatal to animals receiving only the bare minimum necessary for growth

In Cambridge we have been attempting to repeat Meunier s experiments with sodium benzoate, and have confirmed that this substance sometimes causes reduced growth or precipitates xerophthalmia So far we have not succeeded, however, in completely reproducing the balance between benzoate and vitamin A which was observed by the French workers One indication in our limited experience has been that females are more resistant than males to the toxic action of the benzoate

Exposure to Cold

The resistance of rats deficient in vitamin A to low temperatures has been studied by Ershoff (1950) who found that depletion

however, that the factor responsible for the differences between the two groups was not a disparity in their liver reserves but the persistence of undetected injuries in the group which had gone through the period of depletion.

Dr Sharman and I (1951) have studied the effect of cold in a different way, and in several experiments have attempted to cancel out its effect in rats by giving liberal amounts of vitamin A. The results of our most successful experiment are given graphically in Fig. 5. A number of young female rats which had been kept for 5 weeks on a diet deficient in vitamin A, but which were still growing rapidly on account of the presence of fairly large initial reserves were divided into 3 groups each of 6 or 7 animals. One group remained in a heated rat room kept at a temperature of 70° F., but the two other groups had to endure the cold of winter in an unheated shed. One group kept in the cold remained undosed, but the other animals each received 1,000 I.U. of vitamin A daily as halibut liver oil. From the average growth curves it will be seen that the undosed animals kept in the warm room gained considerably in weight during the next few weeks, as also did the animals kept in the cold and dosed with vitamin A. In those kept *in the cold without vitamin A*, however, the increases were insignificant. There was some indication that the fluctuations in weight in the undosed animals and the irregularities in growth in the dosed animals, were influenced by day to day variations in temperature.

These experiments indicate that under carefully chosen conditions it is possible to counteract at least one of the effects of cold by improving the vitamin A status. There is obviously no evidence as yet that vitamin A plays a specific role in influencing resistance to low temperatures.

Maintenance of the Normal Distribution of Vitamin A in the Male

We have already mentioned that vitamin A is poorly absorbed by the liver at low intakes, and have commented on the observations of Johnson and Baumann on the accumulation of vitamin A in the kidney. If we are prepared to regard the shift of emphasis from the liver to the kidney as an abnormality, indicating a mild and temporary

The influence of sex on the distribution of vitamin A is most clearly seen at low levels of dosing, which may be chosen so as to result in the concentration of vitamin A mainly in the kidney in the male, but in the liver in the female The normal predominance of the liver at low levels of dosing may be produced in castrated male animals by injections of oestradiol Increasing the level of dosing with vitamin A, even in normal male animals, causes the concentration in the kidney to be reduced, it appears therefore that at low levels of dosing the liver is less efficient in absorbing vitamin A than at higher levels Reichstein s compound L has been reported to have much the same effect as oestradiol on the distribution of vitamin A at low levels of intake

(3) *Hidden forms of vitamin A*

It has long been known that rats are able to grow in the absence from their tissues of chemically detectable amounts of vitamin A Claims that a hidden form of vitamin A is present in the blood and tissues of albino rats still require confirmation Both French and American workers have advanced evidence of the presence in lard of a concealed form of vitamin A Slight biological activity, not associated with the usual chemical tests for the vitamin, has been claimed to persist in foods which have been fortified with vitamin A and then stored in air Previous evidence that oxidation products of vitamin A may be formed *in vivo* have been supported by spectro scopic observations on extracts from the lungs of rats sacrificed at intervals after massive doses of vitamin A The ability of vitamin A acid to promote growth in rats, and its inability to give rise to the storage of vitamin A in the liver, have both been confirmed Concealed forms of vitamin A, or of provitamin A, have been reported in shrimps and in calanoid copepods

(4) *Benefits derived from a liberal intake of vitamin A*

The substantial vitamin A reserves usually found in well nourished human subjects, and in most wild animals, indicate that they receive

intakes of vitamin A much above the minimal level Claims have been made that such liberal intakes of vitamin A in rats prolong life and increase the resistance to poisons and cold In carefully contrived experiments the effects of cold have sometimes been partially counterbalanced by increasing the allowance of vitamin A A liberal intake of vitamin A, as previously stated, is necessary to preserve the normal distribution of vitamin A between the liver and the kidney in the male rat, and to maintain regular oestrous cycles in females It must not be implied, however, that all these beneficial effects of a liberal intake of vitamin A necessarily indicate a specific interrelation between the vitamin and the particular metabolic stress which is involved

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Discussion

Dr Hickman I wonder if Dr Harris would like to comment on the selection of vitamin A substitutes?

Dr Harris I d like to first of all congratulate Dr Moore on a very fine and comprehensive presentation, references extending from Hippocrates to papers in press—one can't go much further than that There are several points upon which I would like to comment I ll mention first the vitamin factor postulated by Kaunitz We have the feeling in our laboratory from work that we've done trying to confirm the existence of the Kaunitz factor that there are two possible explanations other than the one Kaunitz gives of the results he obtains, first, when one uses the sub optimal or in fact very minimal amounts of vitamin A or carotene which he was dealing with, then the presence of antioxidants or preserving agents becomes very important And we know that the lard distillate which he used is a concentrate of the anti oxidants originally in the lard, and that actually the amount that was present in the supplements fed may have spared enough of the minimal amounts of

the vitamin A so that he could get the results reported Also, secondly, we know the relative inadequacy of the chemical test for determining vitamin A in the lard distillates Perhaps enough vitamin A was present in the tremendous quantities of the lard distillate that he had to give to obtain his results, this small amount of vitamin A merely escaped observation or measurement by the methods used

We've had the opportunity of working with pure vitamin A acid and vitamin A aldehyde and various other vitamin A derivatives and we've also been struck with the evanescent character of vitamin A acid that Dr Moore mentioned One can't find it anyplace in the body after feeding it However, I don't think he mentioned trying to find it in the feces We have found anywhere from 30 to 60 per cent of the vitamin A acid that has been given orally in the feces We have also fed vitamin A acid to animals and found that there was no demonstrable vitamin A, as such or vitamin A acid in the liver and yet when the liver of such animals was subsequently fed to vitamin A deficient animals there was a slight indication of some vitamin A active material

Vitamin A aldehyde is quite interesting physiologically In all respects this derivative of vitamin A seems to satisfactorily substitute and pinchhit for vitamin A itself

I would like to speculate about some of the variability in vitamin A storage that Dr Moore mentioned We know that in cases of stress and strain in animals the vitamin C in the body markedly decreases We also know from McConnell's work that vitamin A follows the same course as vitamin C In physiological stress of many kinds the vitamin A in the body, including the vitamin A in the adrenal goes down to a very low level so that some of the variation which Dr Moore obtained may have been due to this relation between stress and vitamin A It might even explain—harking back to Dr Phillips's paper—the low level of vitamin A in the newborn Perhaps there is normally an equilibrium of vitamin A in the body of the fetus and of the mother but due to the trauma of birth and the stress of changing to air breathing and other shock that the newborn must undergo the newborn infant shows low levels of vitamin A in its liver

Dr Hickman I think that Dr Harris has brought out a very important point namely that when one is doing quantitative measurements of vitamin A rather than qualitative to fail to take into account and to make elaborate analyses of fecal excretion simply leaves you in the dark as to how much vitamin A ever got into the animal to start with and that one certainly needs to know May I have some more comments?

Dr Nilson One thing that may explain the results of the Frenchmen is that the absolute requirements of vitamin A vary with different strains of rats I find that in working with genetically homogeneous strains of rats that one

gets rather wide variations in requirements It may well be that the rats mentioned had a very much lower requirement than the albinos

DR GYORGY Just one short question The low vitamin A level of the blood in pregnancy changes rapidly to normal figures after delivery Is it possible that blood vitamin A is under the influence of the female sex hormones which are abundant during pregnancy?

DR DEUEL I d like to call attention to the sex difference again because it s in line with work that I know many of you will remember that we did on fat many years ago in showing differences in the metabolism of fat as related to sex, not only in the human subjects but also in the rat and in the guinea pig I wouldn t have brought that up except for the fact that we have a paper in press right now on the relationship of the requirement of unsaturated fatty acids in the rat Whereas, we can show that a straight line function obtains between log dose of the linoleate and increase in weight in the male rats up to a level of 200 mg of linoleate per day and possibly higher, when it comes to female rats, 20 mg in one series and 50 mg in another series of experiments were the optimum values for linoleate, further increases in dosage cause a decrease in response In other words, not only in the case of fat but also in such a related substance as unsaturated acids, we have this variation in requirement as related to sex I want to congratulate Dr Moore on going into that point because so many people have reported experiments on rats irrespective of sex and have not even mentioned the sex of the rats that were used

THE BIOCHEMISTRY OF VITAMIN A

George Wald *
Biological Laboratories, Harvard University, Cambridge

The part that vitamin A plays in vision, though of considerable importance, is not its principal activity. It is enough to recall that animals deprived of vitamin A stop growing and eventually die, and neither as the result of night blindness. Vitamin A must play some very general role in cellular metabolism or structure, a role perhaps particularly associated with epithelial cells, since these undergo such marked changes early in vitamin A deficiency.

From this more general viewpoint the visual function of vitamin A seems very wide of the main mark. Yet a paper with the present title must be concerned primarily with this, for it is the only physiological activity of vitamin A that is at all understood.

Fortunately in concentrating upon the visual function we do not wholly abandon the other. Our early work with the chemistry of visual systems made them seem very much isolated, intellectually and materially. When a few years ago we found that one of the main components of the rhodopsin system is cozymase, my coworker exclaimed, "What a relief to connect with the rest of biochemistry."

Since then more connections have emerged that relate the visual processes with those of retinal respiration and fermentation, and with the general metabolism of vitamin A throughout the organism. Reactions of vitamin A have appeared that are in no way confined to the retina, and the pattern of the visual processes has begun to provide a clue to the nature of the reactions in which vitamin A takes part elsewhere in the body.

In this essay I shall review briefly recent work on the chemistry of visual systems, and then discuss the implications of this work for general problems of vitamin A metabolism. I shall try to make

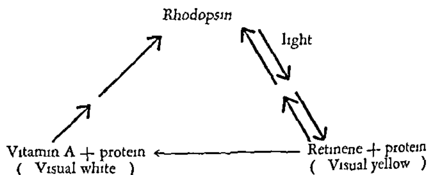
* The recent research reported from our laboratory has been supported in part by the Medical Sciences Division of the Office of Naval Research.

the first purpose serve the second, by laying particular weight upon those aspects of visual metabolism that have consequences outside the retina

RHODOPSIN, PORPHYROPSIN, IODOPSIN

Most vertebrate retinas contain two kinds of light receptor rods, concerned with vision in dim light, and cones, the organs of vision in bright light and color vision The point of attack by light in each of these elements is a photosensitive pigment, rhodopsin or porphyropsin in the rods, iodopsin in the cones All these substances are carotenoid proteins, proteins bearing carotenoid prosthetic groups to which they owe their color and which undergo characteristic changes under the influence of light

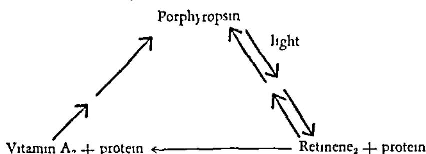
Rhodopsin is a brilliant red pigment, found in the rods of marine fishes and land vertebrates On exposure to light it bleaches over *orange intermediates* (*transient orange*) *to a yellow product* (visual yellow , indicator yellow), and eventually to colorless ness (visual white) Some years ago it was shown that these changes in appearance correspond with chemical changes which are *arranged approximately as follows* (Wald, 1935 36 a, b)



Light bleaches rhodopsin over orange intermediates to a mixture of the yellow carotenoid, retinene and a protein which we now call opsin (Fig 1) Then retinene is converted to vitamin A Both vitamin A and retinene, together with opsin, revert to the visual pigment

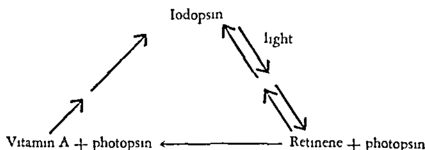
In the rods of freshwater fishes lampreys and certain amphibia—vertebrates which live in or at least originate in fresh water—another

light sensitive pigment is found This is purple in color, and is called porphyropsin It engages in a system of reactions the exact counterpart of the rhodopsin cycle, but involving other carotenoids (Wald, 1937 a, 1938 39)



In every detail that has been examined, the porphyropsin system mimics rhodopsin Certain enzymatic components of both systems are identical, and opsin itself appears to be interchangeable in both systems

Iodopsin, a pigment discovered in the cones of the chicken retina, is involved in a series of reactions which resemble the rhodopsin system even more closely (Wald, 1937 b, Bliss, 1945 46 a, b) Here the carotenoids are identical with those of rhodopsin, it is the protein that is different As in the case of the heme proteins, in which the linkage of a single prosthetic group, iron protoporphyrin, to different proteins determines whether one obtains hemoglobin, or catalase, or peroxidase, so here it is the opsin that decides whether one obtains rhodopsin or iodopsin To make this distinction clear, we shall call the opsins of the rod pigments *scotopsins*, those of the cone pigments *photopsins* The iodopsin system of the chicken retina can then be written (Wald, Brown and Smith, 1952)



Visual systems, therefore, present us with a remarkably homo geneous array of substances, processes and problems When we

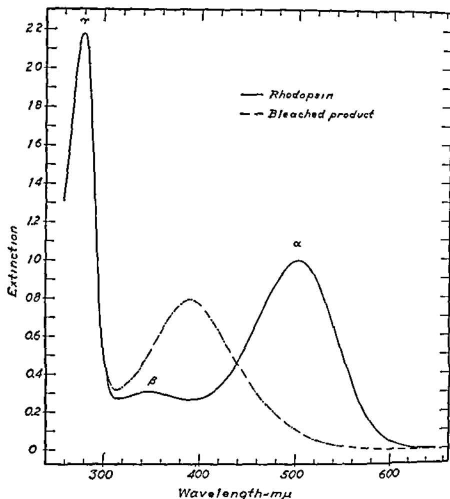


FIG 1 Spectra of rhodopsin and of the product of its bleaching in aqueous digitonin solution Rhodopsin possesses three absorption maxima the α band which is mainly responsible for the spectral sensitivity of rod vision, the β band which together with the α band belongs to the carotenoid prosthetic group and the high γ band, which is due to the protein, opsin On bleaching in solution the α and β bands are replaced by the absorption spectrum of retinene, the protein band remains unchanged (From Wald, 1949a)

have dealt with one, we have very nearly dealt with them all For greater brevity and clarity, I shall consider in detail only the rhodopsin system It should be remembered however that what is said for this pigment applies with little modification to all the others

RETINENE AND VITAMIN A

As already noted, the bleaching of rhodopsin is a complex process (Fig 2) It begins with a typical photochemical reaction, which converts rhodopsin to a highly unstable, orange red product called lumi rhodopsin In the dark, lumi rhodopsin continues to react,

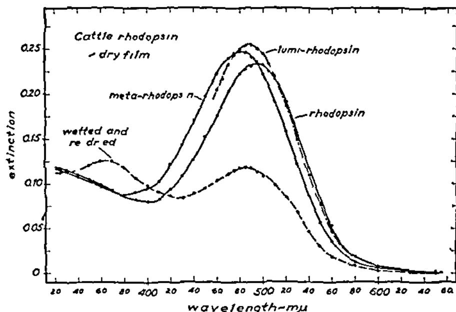
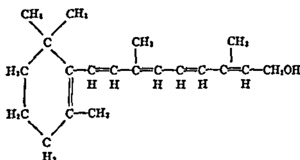


FIG 2 Bleaching of rhodopsin in a dry gelatine film The spectrum was measured in a film dried over calcium sulfate This was then exposed to the momentary illumination of a photoflash lamp and the spectrum immediately remeasured (lumi rhodopsin) After about 1 hour at room temperature in the dark the spectrum had changed further (meta rhodopsin) These changes were complete a second exposure to a photoflash lamp produced no further effect The film was then soaked in m/15 *neutral phosphate buffer for 10 minutes and redried all in the dark* The spectrum of the final product shows a mixture of regenerated rhodopsin and of retinene + opsin in roughly equal amounts On renewed exposure to light such regenerated rhodopsin goes through another cycle of the same changes (From Wald Durell and St George 1950)

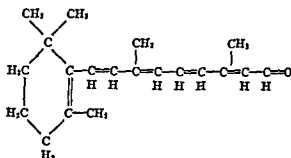
going over with little change in color to meta rhodopsin Then, given access to water, meta rhodopsin bleaches in the dark to a mixture of retinene and opsin (Wald, Durell and St George, 1950)

We owe the identification of retinene to Morton and his coworkers

Vitamin A is the primary alcohol, $C_{19}H_{27}CH_2OH$ Morton has shown that retinene is vitamin A aldehyde, $C_{19}H_{27}CHO$ (Ball, Goodwin and Morton, 1948)



Vitamin A, $C_{19}H_{27}CH_2OH$



Retinene, $C_{19}H_{27}CHO$

Morton showed also how to prepare retinene by the mild oxidation of vitamin A His simplest procedure was to add a pinch of manganese dioxide powder to a solution of vitamin A in petroleum ether, and to leave this in the refrigerator for several days At the end of this period the vitamin A had been converted almost entirely to retinene (Ball, Goodwin and Morton, 1948)

On re examining this reaction, we found it to take the following course Vitamin A is adsorbed very strongly on manganese dioxide, and in the adsorbed state is oxidized to retinene Retinene, however, is much less strongly adsorbed, and so is displaced from the manganese dioxide surface by the remaining vitamin A as fast as it is formed In this way all the vitamin A passes over the surface of the adsorbent, and is replaced by retinene in the solution (Wald, 1947-48)

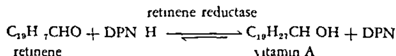
With this mechanism understood, the process was modified accordingly. All that is needed is to pack a small amount of manganese dioxide powder—about 0.6 g. to oxidize 10 mg. of vitamin A—into a piece of glass tubing, so as to form a short column of the kind used in chromatography. A solution of vitamin A in petroleum ether is poured in at the top, and is drawn through under light suction. An almost pure solution of retinene runs off as the filtrate (Wald, 1947–48).[1]

I have called this process a *chromatographic oxidation*, and have suggested that it provides an example of a widespread and important class of reactions, in which a solid acts at once as adsorbent and reagent. Such processes probably display a degree of specificity and molecular orientation to be found otherwise only in enzyme reactions. The force of this consideration will appear in connection with another such process below.

The Alcohol Dehydrogenase System

We have described the oxidation of vitamin A to retinene. In the retina one observes just the reverse process, the virtually quantitative reduction of retinene to vitamin A.

This process is catalyzed by a soluble enzyme, which we have called retinene reductase. It works together with the major coenzyme of biological oxidations and reductions: cozymase, or DPN, the substance which in yeast reduces acetaldehyde to ethyl alcohol, and in muscle reduces pyruvic to lactic acid. In the retina it transfers two hydrogen atoms to retinene, reducing its aldehyde group to the alcohol group of vitamin A (Wald and Hubbard, 1948–49).



$$\underset{\text{retinene}}{C_{19}H_{27}CHO} + DPN \cdot H \xrightleftharpoons{\text{retinene reductase}} \underset{\text{vitamin A}}{C_{19}H_{27}CH_2OH} + DPN$$

This system is readily assembled in solution (Wald, 1949 b,

[1] Mr. Paul Brown in our laboratory has recently found that the addition to the vitamin A solution in petroleum ether of about 2 per cent absolute ethyl alcohol results in a purer product and an increased yield.

1950) The enzyme-protein or apoenzyme is extracted from whole retinas or from suspensions of the outer segments of the rods with buffer solutions Reduced cozymase is prepared by Ohlmeyer's procedure (1938) As substrate, retinene is prepared by the chromatographic oxidation of vitamin A on manganese dioxide as described above When these three components are mixed, the retinene is almost entirely reduced to vitamin A (Fig 3, upper portion)

Cozymase introduces a second vitamin into the chemistry of vision, for its active constituent is nicotinamide, the anti pellagra factor of the vitamin B complex In the retina it is in the peculiar position of helping to regenerate vitamin A

Indeed it is helpful on occasion to add a third vitamin to this system *in vitro* Tissue homogenates and tissue extracts frequently contain a so called nucleosidase, an enzyme which destroys cozymase The coenzyme can be protected from this attack by adding free nicotinamide (Mann and Quastel, 1941, Handler and Klein, 1942) or alternatively α tocopheryl phosphate (Spaulding and Graham, 1947) In the latter instance, one is treated to the strange spectacle of three vitamins cooperating in a single chemical reaction in solution —vitamin E phosphate protecting a niacin complex as it reduces retinene to vitamin A

The same enzyme system reduces retinene$_2$ to vitamin A$_2$ (Fig 3, lower portion) Cozymase is again the coenzyme, and the enzyme proteins are interchangeable between the rhodopsin and porphyropsin systems The substrate in this instance is retinene$_2$, prepared by the chromatographic oxidation of vitamin A$_2$ on manganese dioxide (Wald, 1950)

Both the rhodopsin and the porphyropsin systems therefore make use of a single enzyme, which with reduced cozymase as coenzyme, reduces either of the retinenes to the corresponding vitamin A

Th field of activity of this enzyme however appears to range far beyond the two retinenes, and indeed far beyond the retina It is well known that a variety of animal tissues—liver, kidney, intestine—contain the enzyme, alcohol dehydrogenase, which together with cozymase catalyzes the equilibrium between ethyl and higher alcohols and the corresponding aldehydes (Lutwak Mann, 1938) Shortly

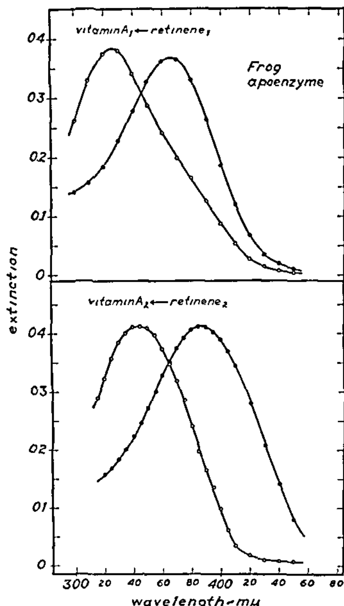


FIG 3 The action of alcohol dehydrogenase from the frog retina on retinene₁ and retinene₂ Each preparation included retinene dissolved in 1 per cent digitonin solution 0 7 mg of reduced cozymase per ml 5 5 mg of nicotinamide per ml, and an extract of homogenized frog retinas in m/30 phosphate buffer, pH 6 81 The controls differed only in that the retinal extracts were replaced either with the same extract which had been boiled for one half minute (upper figure) or with the phosphate buffer alone (lower figure) The experimental and control mixtures were incubated for 2 hours at 23° C Methanol was added to each to a concentration of 60 per cent and they were extracted with hexane The spectra of the hexane extracts are shown Those from the controls (solid circles) show the unaltered retinenes, those from the enzyme mixtures (open circles) show complete reduction to the corresponding vitamins A (From Wald 1950)

after the discovery of the retinene reductase system in the retina, Bliss (1949) reported that crude preparations of alcohol dehydrogenase from rabbit liver catalyze the equilibrium between retinene and vitamin A We have confirmed this observation with the crystalline alcohol dehydrogenase prepared from horse liver by Bonnichsen (1950) Warren Yudkin has also shown in our laboratory that the frog retina can oxidize ethyl alcohol to acetaldehyde (Hubbard and Wald, 1951, Wald, 1951)

What we have called retinene reductase therefore has properties very much like the alcohol dehydrogenase of animal tissues The two enzymes may indeed be identical We have up to now called them by different names because their identity has not been demonstrated, but it might be more economical to call them by one name until they have been shown to be different I shall adopt this practice in what follows, and refer to both the retinal enzyme and that found in other animal tissues as alcohol dehydrogenase

This is the only enzyme yet known to react directly with vitamin A It ushers in a broad series of relationships between the visual processes and other metabolic systems Through cozymase it connects the metabolism of vitamin A with the main pathways of cellular respiration and fermentation, in which cozymase plays a central role In alcohol dehydrogenase a wide variety of tissues share an enzyme which permits them to negotiate reversible transformations between vitamin A and retinene This enzyme fills such an important function in the retina, and is likely to take such an important position in the whole of vitamin A metabolism, that its properties deserve careful examination

Two alcohol dehydrogenases are known, which with cozymase govern the equilibrium between ethanol and acetaldehyde One we have already considered in part, the other, found in yeast, has been crystallized by Negelein and Wulff (1937) and by Racker (1950)

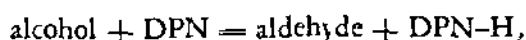
Unlike the animal enzyme, yeast alcohol dehydrogenase is not a catalyst for the vitamin A retinene equilibrium (Bonnichsen, personal communication, cf Hubbard and Wald, 1951, Bliss, 1951) Yeast alcohol dehydrogenase also is inhibited by monoiodoacetate, the animal enzyme is not For this reason it is usually stated that

the yeast enzyme, unlike that of animal tissues, depends for its action upon sulfhydryl (-SH) groups.

We have recently found however that crystalline liver alcohol dehydrogenase is inhibited completely by the more powerful and specific sulfhydryl poison, p-chloromercuribenzoate, in concentrations of about 2×10^{-4} M. This inhibition is reversed with glutathione. There is no doubt therefore that animal alcohol dehydrogenase, like that of yeast, is a sulfhydryl enzyme (Hubbard and Wald, unpublished observations).

On the basis of these observations, Theorell and Bonnichsen (1951) have examined the mechanism of the sulfhydryl effect. They had found earlier that crystalline liver alcohol dehydrogenase forms a complex with reduced cozymase, as a result of which the absorption band of the coenzyme, normally at 340 mμ, is shifted to 325 mμ. They now observed that on adding p-chloromercuribenzoate to this complex, the absorption immediately shifted back to 340 mμ. It seems therefore that sulfhydryl groups of alcohol dehydrogenase are concerned with binding cozymase to the enzyme. Whether they are also concerned with binding retinene is still to be determined. We have already shown that sulfhydryl groups of opsin are involved in binding retinene to form rhodopsin (Wald and Brown, 1951, 1951-52), and it is very possible that such groups also govern the linkage between retinene and alcohol dehydrogenase.

The equilibrium instituted by the alcohol dehydrogenase system, whatever the specific substrates involved, can be written:



$$\text{alcohol} + \text{DPN} \rightleftharpoons \text{aldehyde} + \text{DPN-H}_2$$

For this one can write the mass action expression:

$$K = \frac{(\text{aldehyde})\ (\text{DPN-H}_2)}{(\text{alcohol})\ (\text{DPN})}$$

Racker (1950) has measured the ethanol-acetaldehyde equilibrium catalyzed by crystalline yeast alcohol dehydrogenase. He found the constant K to have the value 1.3×10^{-4} at pH 7. In consequence, in a system in which cozymase is half reduced, half oxidized, the ratio of alcohol to acetaldehyde should be about 8000 : 1. Or if

equimolar quantities of alcohol and DPN are mixed, then when equilibrium is established the ratio of alcohol to acetaldehyde should be about 90 1 These calculations express the fact that the equilibrium in this reaction lies far over toward the side of reduction toward the alcohol

Racker has found however that the ethanol acetaldehyde equilibrium depends markedly upon pH (Fig 4) The logarithm of the equilibrium constant, log K, varies linearly with pH between about pH 7 and 9 6 The reason for this variation is apparent if one rewrites the equilibrium as follows



$$\text{alcohol} + \text{DPN}^+ = \text{aldehyde} + \text{DPN–H} + \text{H}^+$$

for which the mass action expression becomes

$$K_H = \frac{(\text{aldehyde})\ (\text{DPN–H})\ (\text{H}^+)}{(\text{alcohol})\ (\text{DPN}^+)}$$

The new equilibrium constant, K_H, is independent of pH For the ethanol acetaldehyde equilibrium it has the value $1\ 15 \times 10^{11}$ (Racker, 1950)

The net result of this discussion is that increase in alkalinity markedly displaces the alcohol aldehyde equilibrium in the oxidative direction, toward the formation of aldehyde At pH 7 the ratio of ethyl alcohol to acetaldehyde when DPN is half oxidized, as already stated is about 8,000, at pH 8 it has fallen to about 800, at pH 9 to about 80 This may be an important consideration in certain types of physiological system

Bliss (1951) has examined the equilibrium between retinene and vitamin A from this point of view, using a purified preparation of alcohol dehydrogenase from horse liver (Fig 4) He has found an equilibrium constant, K, which depends upon pH just as described above, and again a K_H can be computed which is independent of pH The vitamin A retinene equilibrium however lies very much further over toward the oxidative side—toward retinene—than those described by Racker K_H is about $3\ 3 \times 10^{9}$, and at pH 7 K is about $3\ 5 \times 10^{2}$ In this case therefore, in an equilibrium mixture in which cozymase is half oxidized, the ratio of vitamin A to retinene at pH 7

is about 30 1 Alternatively, if equimolar amounts of vitamin A and DPN are mixed, at equilibrium the ratio of vitamin A to

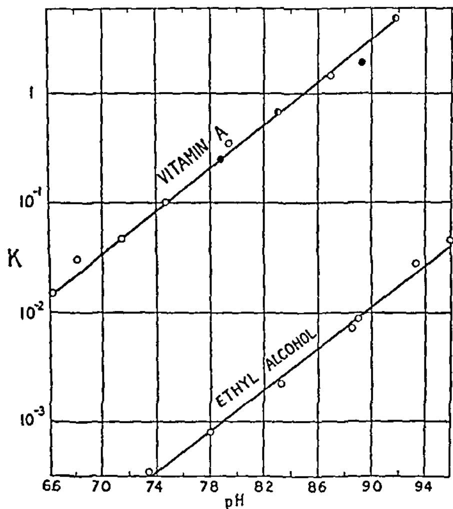


FIG 4 The vitamin A retinene and the ethanolacetaldehyde equilibria as a function of pH Ordinates show values of the equilibrium constant K The data on the vitamin A retinene equilibrium are from Bliss (1951) and involve the action of horse liver alcohol dehydrogenase The data on ethyl alcohol are from Racker (1950) and involve the action of crystalline yeast alcohol dehydrogenase (From Bliss 1951)

retinene should be about 5 4 1 At more alkaline pH s the equilibrium favors retinene formation still further, conversely at pH 6, having started with equal amounts of vitamin A and DPN, the equilibrium ratio of vitamin A to retinene would be about 17 1

A final consideration may help to sway this equilibrium toward retinene Theorell and Bonnichsen (1951) have observed that the alcohol dehydrogenase cozymase complex has a considerably higher oxidation reduction potential than free cozymase In the presence of *high concentrations of enzyme, favoring the formation of the* complex, a much stronger oxidation of alcohol to aldehyde is anticipated Theorell and Bonnichsen state that in liver the molar concentration of the enzyme approaches that of DPN, ensuring a nearly complete binding of the coenzyme at neutrality

All these factors together make it appear that though the vitamin A retinene equilibrium established by animal alcohol dehydrogenase favors the reduction of retinene to vitamin A, the disproportion is not extreme, and it would require little in the way of special conditions to unbalance the system in the other direction, to promote the oxidation of vitamin A to retinene We shall see that this consideration is of the greatest significance for the visual processes, and that it may play a part in connection with other phases of vitamin A metabolism

This view of the vitamin A retinene equilibrium leaves somewhat to be explained why in the isolated retina, retinene is reduced so completely to vitamin A that ultimately no retinene can be distinguished Probably the acidification of the tissue by metabolic products, which as we have seen inclines the equilibrium toward the reductive side, has some part in this In addition this behavior may be promoted by a process not yet mentioned, the esterification of vitamin A This seems to go on with great activity in the retina, and may, by removing vitamin A, displace its equilibrium with retinene continuously in the direction of reduction

The Synthesis of Rhodopsin

We have described the degradation of rhodopsin to a mixture of vitamin A and opsin In the intact eye these products revert to form the visual pigment

Many years ago Kuhne recognized that rhodopsin is synthesized in the retina in two ways a relatively rapid anagenesis from

yellow precursors, which occurs to some extent in the isolated retina and even in solution, and a much slower neogenesis from colorless precursors, which Kuhne believed to occur only in the living eye These processes can now be identified with the synthesis of rhodopsin from retinene, and from vitamin A

Recently Hecht *et al* (1936) and Chase and Smith (1939 40) confirmed Kuhne s observation that rhodopsin regenerates to a small extent, after bleaching in solution to retinene and opsin The largest regeneration reported was about 15 per cent

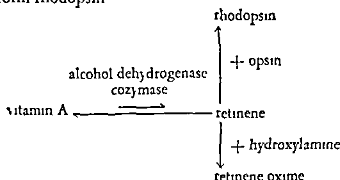
We have found that when such solutions are supplemented with retinene they regenerate up to 85 per cent of their original content of rhodopsin after a first bleaching (Wald and Brown, 1950) Furthermore, one can prepare from wholly bleached rods a colorless, carotenoid free solution of opsin When this is incubated with retinene in the dark, it forms a high concentration of rhodopsin (Fig 5)

This reaction does not require the presence of any molecules other than retinene and opsin What is more surprising, it requires no external source of energy It is a spontaneous—i e, an energy yielding—reaction It is the *bleaching* of rhodopsin that demands energy, usually provided by light Given the opportunity, retinene and opsin reunite spontaneously to regenerate the visual pigment

There remains the more difficult problem, the synthesis of rhodopsin from vitamin A This appeared so much more involved and to have altogether such special properties that I had assumed with Kuhne that it goes by a special route Yet the ease with which retinene and opsin unite to form rhodopsin suggested that if it were only possible to oxidize vitamin A to retinene the job would be done This indeed proved to be the case All the difficulty in making rhodopsin from vitamin A centers in the difficulty of oxidizing vitamin A to retinene

We have already noted that the equilibrium between vitamin A and retinene favors reduction rather than oxidation In the very much more unbalanced equilibrium between ethanol and acetaldehyde, however, it has long been known that the reaction can be driven in the oxidative direction by introducing an aldehyde trapping

The retina, however, already contains a specific retinene trapping reagent in opsin Opsin should be able to substitute in the alcohol dehydrogenase system for hydroxylamine, and so drive a continuous oxidation of vitamin A to retinene, by continuously condensing with retinene to form rhodopsin



The trouble with this notion is that an isolated retina, bleached to colorlessness, presumably contains all the components needed to make rhodopsin in this way, yet when replaced in the dark it does not visibly produce rhodopsin

Nevertheless, on careful extraction of whole frog retinas which had been bleached to colorlessness and then incubated in the dark, it turned out that they do form a little rhodopsin, perhaps 10 per cent as much as would be formed during dark adaptation *in vivo* Retinal homogenates behave similarly On supplementing retinal homogenates with cozymase the yield of rhodopsin was doubled Kuhne had believed that the synthesis of rhodopsin from colorless precursors—i e, from vitamin A—demands the cooperation of the pigment epithelium, and we found that the addition to retinal homogenates of a homogenate of pigment epithelium doubles the yield of rhodopsin again, bringing it to about 40 per cent (Wald and Hubbard, 1950)

What the pigment epithelium contributes to this synthesis is not yet wholly analyzed One factor which it is known to contribute, however, is vitamin A A frog retina homogenate can be shown to make rhodopsin from vitamin A supplied by a pigment epithelium homogenate Alternatively, the addition of free vitamin A to a retinal homogenate increases considerably the yield of rhodopsin

Another factor which helps is the addition of respiratory enzymes—for example the particulate 'succinoxidase' system of pig heart—which presumably aid by keeping cozymase oxidized When all these types of supplementation are combined, we obtain yields of rhodopsin as high as 60 per cent (Hubbard and Wald, 1951)

All the factors therefore which promote the oxidation of vitamin A to retinene aid the synthesis of rhodopsin The trapping of retinene by opsin, so to speak, draws it from before, while simultaneously

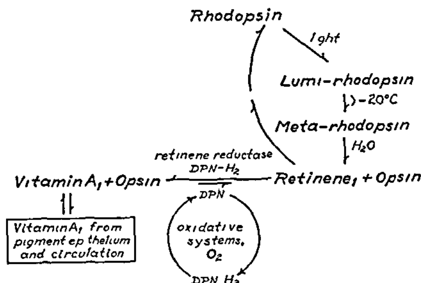


FIG 6 Reactions of the rhodopsin system The bulk of the system lies within the outer segments of the retinal rods but it is supplemented with vitamin A respiratory factors and oxygen itself from the pigment epithelium and the blood circulation (From Hubbard and Wald 1951, Wald 1951)

it is pushed from behind by the addition of vitamin A, cozymase, the oxidant of vitamin A, and respiratory enzymes which keep cozymase oxidized

The rhodopsin system therefore can be formulated as in Fig 6 What we had originally supposed to be a special pathway for the synthesis of rhodopsin from vitamin A now appears to consist in the special conditions needed to drive the oxidation of vitamin A to retinene

If this conception of the rhodopsin system is correct, it should be possible to assemble the system in solution by mixing four substances

vitamin A, cozymase, alcohol dehydrogenase and opsin This is indeed true We have made up such mixtures with vitamin A from fish liver oil, cozymase from yeast, and crystalline alcohol dehydrogenase from horse liver Only opsin needs to be obtained from the

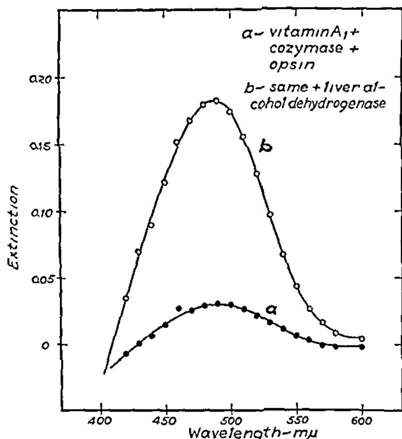


Fig 7 Synthesis of rhodopsin in a solution of known components The upper curve shows the difference spectrum—the difference in absorption spectrum before and after bleaching—of rhodopsin synthesized by incubating together vitamin A from fish liver oils crystalline horse liver alcohol dehydrogenase cozymase and cattle opsin The lower curve shows the rhodopsin formed in an identical mixture lacking only the alcohol dehydrogenase (From Hubbard and Wald 1951)

retina When such a mixture is placed in the dark, it synthesizes rhodopsin (Fig 7) Brought into the light, it bleaches Replaced in the dark, it makes more rhodopsin This mixture of four substances, therefore, carries out in solution all the reactions of the rhodopsin system

tion therefore appeared to mean that crystalline, all-trans vitamin A can not serve as a precursor of rhodopsin, but that this pigment requires for its synthesis a cis-isomer of vitamin A present in liver oil

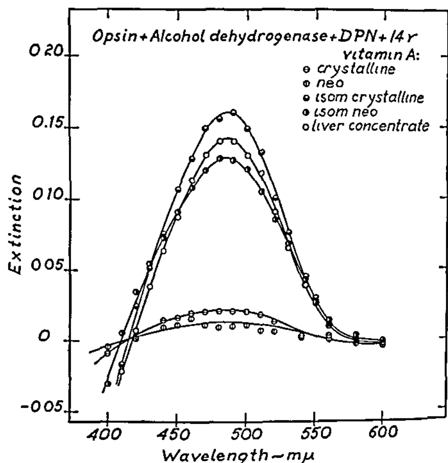


FIG 9 The synthesis of rhodopsin from vitamin A of five different origins crystalline all trans, crystalline neovitamin A, isomerized all trans, isomerized neo, and fish liver oil concentrate The figure shows the difference spectra of rhodopsin obtained by incubating equal amounts of each of these preparations with opsin, alcohol dehydrogenase, and cozymase Only traces of rhodopsin were synthesized from all trans or neovitamin A, but after these substances had been isomerized with light in the presence of iodine, they were as effective as liver oil vitamin A in forming rhodopsin (From Hubbard and Wald, 1952, 1952-53)

Zechmeister (1944) has shown that carotenoids are isomerized by irradiation with light in the presence of a trace of iodine When all-trans vitamin A was treated in this way, it became as efficient a precursor of rhodopsin as the liver oil vitamin (Fig 9). The same

was true of crystalline neovitamin A, a sample of which was sent us by Dr Baxter It yielded almost no rhodopsin until irradiated in the presence of iodine (Fig 9) The synthesis of rhodopsin therefore demands some cis isomer of vitamin A other than neovitamin A

This synthesis, it will be recalled, proceeds in two stages first the oxidation of vitamin A to retinene by the alcohol dehydrogenase system, then the condensation of retinene with opsin to form rhodop sin Which of these steps is isomer specific?

A preliminary examination of the action of alcohol dehydrogenase upon all trans vitamin A and its isomerate shows that this enzyme reacts well with both forms of vitamin A and with the corresponding retinenes Indeed it seems to favor slightly the all trans isomer Clearly this reaction does not govern the isomer specificity of rho dopsin synthesis This is hardly surprising, for alcohol dehydro genase is a very unspecific enzyme which seems to be concerned primarily with the presence of the terminal alcohol or aldehyde grouping

The dominant isomer specific step in the synthesis of rhodopsin is the condensation of retinene with opsin This hardly goes at all *with crystalline all trans retinene, or with neoretinene a, crystallized* by Mr Robert Gregerman in our laboratory, and since by the group which includes Embree and Baxter, at the research laboratories of Distillation Products Industries in Rochester The Distillation Products group has also recently succeeded in crystallizing an isomer which they believe to be the 3 cis isomer of retinene (what we call here isoretinene *a*) When mixed in the dark with opsin, it yields a very lively synthesis of photosensitive pigment, but this is not true rhodopsin Its absorption spectrum is displaced about 15 mμ toward shorter wavelengths, the maximum lying at about 487 mμ (Fig 10)

It had been noted earlier that rhodopsin regenerated in solution usually possesses an absorption spectrum displaced somewhat toward shorter wavelengths (Chase and Smith, 1939 40, Collins and Morton, 1950, Wald, Durell and St George, 1950) Morton has suggested for this material the name iso rhodopsin We have found that it is in fact a *mixture of native and altered forms of rhodopsin* (Wald,

1951) It seems probable now that what should be called iso-rhodopsin is the altered species of rhodopsin synthesized from isoretinene *a*

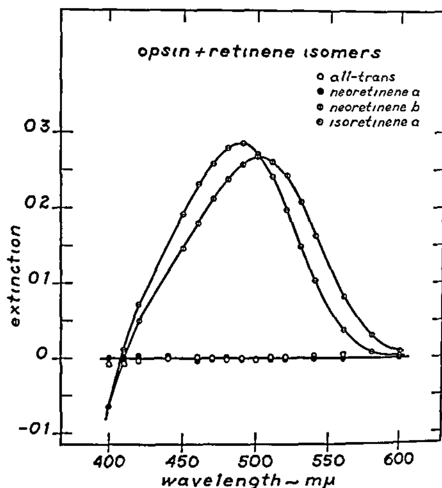


FIG 10 The synthesis of rhodopsin from stereoisomers of retinene In each case an excess of retinene was incubated in the dark with cattle opsin in solution All trans and neoretinene *a* yield no rhodopsin A second mono cis retinene isoretinene *a* yields a light sensitive pigment with an absorption spectrum like that of rhodopsin but displaced about 15 mμ toward shorter wavelengths (iso rhodopsin) Still another mono cis isomer neoretinene *b* yields rhodopsin indistinguishable from that obtained from the retina

Very recently, the Distillation Products group sent us a fourth isomer of retinene in crystalline form, apparently the di cis isomer When mixed with opsin and incubated in the dark, this behaves very

peculiarly There is no rapid reaction such as displayed by an active isomer of retinene, but very slowly a light sensitive pigment forms which again is not rhodopsin, but iso rhodopsin, just as is formed from isoretinene *a* We think that the highly unstable di cis isomer is itself inactive, but when incubated at room temperature in the dark it spontaneously isomerizes to the isoretinene *a* Before this can isomerize further it is picked up by opsin to make iso rhodopsin

We would therefore appear to have reached an impasse, in that all four stereoisomers of retinene which according to present theory can exist in substantial amounts have now been tried in the synthesis of rhodopsin, and all have been found wanting

All of the inactive isomers, however, on irradiating with light in solution, even without addition of iodine, isomerize to yield highly active preparations Dr Ruth Hubbard has recently fractionated an isomerate of all trans retinene by chromatographic adsorption upon acid washed alumina She has obtained a highly active fraction, not yet crystalline but in a high state of purity, which when mixed with opsin forms true rhodopsin indistinguishable from the native pigment (Fig 10)

Judging by this behavior and its absorption spectrum, which seems distinctly different from those of the other retinene samples available to us, this seems to be a fifth stereoisomer of retinene The position of its spectrum which is close to that of neoretinene *a* and isoretinene *a*, indicates that it is probably also a mono cis variety Momentarily we designate it neoretinene *b* The spectrum possesses an unusually strong cis peak (cf Zechmeister 1944) theoretically the sign of a highly bent molecule In some ways it fits the 3 cis configuration shown in Fig 8 better than does isoretinene *a* The existence of what appear to be five stereoisomers of retinene may demand some revision of the present theory of cis trans isomerization in this class of compound

So much for the specific configuration of retinene required to synthesize rhodopsin When rhodopsin is bleached, retinene emerges as one of the products What is its configuration?

We find that the retinene obtained by bleaching rhodopsin is an

inactive isomer Its behavior on isomerization by light shows that it is in all probability the all trans form This must be isomerized before it can re enter the structure of rhodopsin

Retinene therefore goes into rhodopsin as one stereoisomer, and *comes out another For this reason the stereoisomerization of reti* nene, or of the corresponding vitamin A, is an integral and necessary component of the rhodopsin system In cognizance of this process, the system can now be formulated as in Fig 11

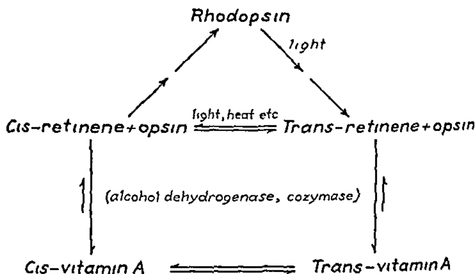


FIG 11 Cis trans isomers of retinene and vitamin A in the rhodopsin cycle Retinene enters rhodopsin as a cis isomer (neoretinene *b*) and emerges all trans This can be re isomerized to the active form by light heat and perhaps other means More generally *in vivo* the all trans retinene is reduced to all trans vitamin A and this is probably returned to the blood circulation while new supplies of the active cis isomer of vitamin A are withdrawn from the circulation to regenerate rhodopsin (From Hubbard and Wald 1952)

Retinene, as already noted, is isomerized on irradiating with visible light Some stereoisomerization of retinene may be expected to occur by this means in the retina itself There is little opportunity ordi *narily, however, for light to act on retinene in the eye,* for it is usually reduced to vitamin A almost as rapidly as formed

The major process by which the retina is kept supplied with the active isomer, therefore, is probably exchange with the circulation What is exchanged is not retinene, but vitamin A The retina continually returns to the blood the all trans vitamin A produced

in the visual process, and takes from the circulation new supplies of the active neovitamin A*b* In this way the visual process is kept intimately connected with the supply and transport of vitamin A throughout the organism

The General Metabolism of Vitamin A

In the preceding pages I have reviewed the way in which vitamin A, in the course of its visual reactions, is linked with the supply, transport and metabolism of this vitamin throughout the body, and with the basic processes of respiration and fermentation in the retina I should like now to approach another kind of question—the nature of the reactions in which vitamin A participates elsewhere in the organism, and the degree to which the retinal processes yield an insight into them Little is known as yet of such reactions, and one can hope to do no more than bring this little together, and indicate directions in which further inquiry may prove fruitful

It has lately been shown that retinene, fed to rats by mouth or injected subcutaneously or intraperitoneally, is reduced to vitamin A as it is absorbed (Glover, Goodwin and Morton, 1948) The gut wall and subcutaneous tissues seem to accomplish this reduction directly, the site of reduction of intraperitoneal retinene is unknown

If the reduction of retinene involved a specific enzyme, the occurrence of this process in the tissues would imply that they encounter retinene in the course of their normal metabolism We have seen however that retinene is reduced by the very unspecific and ubiquitous enzyme alcohol dehydrogenase, whose existence in the tissues is explained adequately by their exposure to a number of other metabolic aldehydes, and makes no special case for retinene as a normal substrate If therefore an argument is to be made for the occurrence of retinene outside the retina, it must be on other grounds

One such possibility, suggested also by Glover *et al* (1948), is that retinene is produced by the oxidation of β carotene In the past few years it has been shown repeatedly that laboratory procedures for oxidizing β carotene yield some retinene among other products Hunter and Williams (1945) obtained very small yields of retinene

(0 4–0 5 per cent) on oxidizing β carotene with hydrogen peroxide in chloroform acetic acid More recently Wendler, Rosenblum and Tishler (1950) obtained as much as 30 per cent retinene by oxidizing β carotene with hydrogen peroxide osmium tetroxide

A special interest attaches to the report of Meunier *et al* (1950, Meunier, 1951) that retinene appears in very high yield when β carotene is oxidized on solid manganese dioxide In this procedure, carotene is dissolved in ethyl ether containing 2 to 3 per cent ethyl alcohol On agitating this solution for about 20 minutes with manganese dioxide powder, using about 100 times as much powder by weight as carotene, and filtering, one is said to obtain about a 60 per cent yield of retinene

It will be recalled that this is essentially the procedure used earlier to oxidize vitamin A to retinene (Ball, Goodwin and Morton, 1948, Wald, 1947 48) I have pointed out that this type of 'chromatographic oxidation,' in which a solid is at once adsorbent and reagent, and acts upon the substrate in the adsorbed state, can be expected to mimic on occasion the specificity and directedness of an enzyme reaction I think that Meunier s observations are a stimulus in this direction, and that they encourage the hope that the tissues contain an enzyme system which oxidizes β carotene with the same effect

Let us for the moment suppose that such an enzyme exists—a carotene oxidase capable of oxidizing β carotene to retinene Then together with alcohol dehydrogenase and dihydrocozymase to reduce retinene, this system would carry out the complete conversion of β carotene to vitamin A

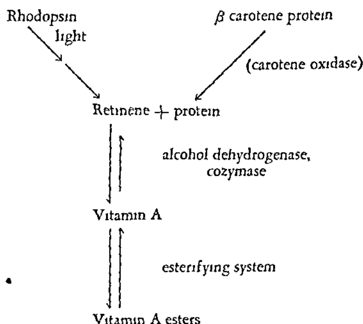
Here I should like to introduce another consideration β carotene, a hydrocarbon, is insoluble in aqueous solutions The same of course is true also of retinene and vitamin A How are such substances brought into the same phase with an enzyme system? How indeed are they transported?

The answer to the latter question is that in the blood, carotenoids probably including vitamin A, are bound to plasma proteins (cf Zechmeister, 1937, p 157) Many years ago Palmer and Eckles (1914) showed that in cattle serum β carotene is bound in a complex with protein which they called 'caroto albumin" In the blood serum

vitamin A The vitamin A of the blood plasma is predominantly the free alcohol, while that of the liver is overwhelmingly esterified (Gray, Hickman and Brown, 1940, Kascher and Baxter, 1945) Glover *et al.* (1948) and Eden and Sellers (1950) found evidence of an active esterification of vitamin A in the intestinal wall and in subcutaneous tissues It can be added that vitamin A seems to be esterified rapidly in the eye tissues The vitamin A of both the retina and the pigment epithelium, as we have found in partition experiments with extracts from frog and cattle eyes, is predominantly in the ester form

When all these processes are brought together, they come to the following view of vitamin A metabolism β carotene, possibly in water soluble combination with a protein, is oxidized by a carotene oxidase to retinene This is reduced by the alcohol dehydrogenase system to vitamin A, and this in turn is esterified in the tissues

The curious thing about this view of events is that it brings the general metabolism of vitamin A into such close relation with the visual processes In both cases a carotenoid protein is degraded over retinene to vitamin A, and this then esterified The parallelism between both types of systems is plain in such a diagram as follows



At present the principal usefulness of such a diagram is to pose a series of distinct problems What is the nature of the combination of β carotene—a hydrocarbon bearing no obviously active groups—with protein? Is it true that such complexes represent the metabolic form of carotene, and perhaps also of vitamin A? Does there exist in the tissues a 'carotene oxidase' that acts as postulated here? What is the enzymatic mechanism which esterifies vitamin A?

Were all these matters settled, they would dispose only of what we may call the vegetative metabolism of vitamin A They would leave untouched the much more interesting questions involving the utilization of vitamin A in the tissues Perhaps here also something is to be learned from the utilization of vitamin A in the retina The widespread distribution of the alcohol dehydrogenase system makes it possible for many tissues to convert vitamin A to retinene, particularly in situations in which some receptor molecule is available to condense with and remove retinene from the system The particular gain in oxidizing vitamin A to retinene as a first step in its utilization is that retinene is an enormously more active molecule It condenses spontaneously with amino and sulfhydryl groups on proteins and other types of molecules undergoes addition reactions of various kinds, and exhibits in general the wide variety of reactions that go with the carbonyl group, particularly when, as in this case, it is a conjugated carbonyl group It is quite possible that in the synthesis of rhodopsin we have a model for a general class of reactions in which vitamin A, through intermediate conversion to retinene, is attached to other molecules to form the complexes upon which its general cellular activities depend

All these reactions must occur under fundamental restrictions imposed by stereoisomeric form In all enzymic reactions, this must constitute a basic consideration, for cis trans isomerization affects the *shape* of the molecule, and to an enzyme *shape* is all important (cf Zechmeister, 1949) We may take it for granted, particularly after the experience gained with visual systems, that the cis trans isomers of all the carotenoids behave more or less differently toward all enzymes or other proteins with which they react

It is somewhat surprising nevertheless that stereoisomeric differ

ences can be demonstrated also in the overall metabolism of the carotenoids The pro vitamin A activity in rats of numerous carotenoid isomers prepared in Zechmeister s laboratory has been estimated by Deuel and his coworkers (Zechmeister, 1949) The stereoisomers of a single carotenoid (e g , α carotene) were found to vary in activity by as much as 400 per cent In general the all trans isomer is most active, though pro γ carotene, a poly cis form, is about 60 per cent more effective in the rat than all trans γ carotene

As for vitamin A, there is good evidence that it stereoisomerizes in the body To a first approximation neovitamin A (Robeson and Baxter, 1947) and the mono cis retinene synthesized by Graham *et al* (1947) yield bioassays comparable with all trans vitamin A in growth tests on rats After feeding neovitamin A, it has been shown also that mixtures of all trans and cis vitamin A are deposited in the liver (Robeson and Baxter, 1947) The rate at which vitamin A stereoisomerizes in the body is not known, but it seems at least to keep pace with such long term processes as growth Even this is not altogether true, however, for on re examining data which had accumulated over a seven year period, Harris *et al* (1951) have concluded that neovitamin A is only 80 7 per cent as effective as the all trans isomer in stimulating growth in rats, and 71 5 per cent as effective in causing the deposition of vitamin A in the liver

One way to stereoisomerize carotenoids is simply to warm them In the neighborhood of 60° C most of them go over to an equilibrium mixture of stereoisomers within 1 to 2 hours (Zechmeister, 1944) What takes this time at 60° C might take a day at mammalian body temperature Perhaps this is all that is involved in the stereoisomerization of carotenoids *in vivo*

These are some of the problems that lie before us Some of them are clearly defined, others—notably those concerned with the general functions of vitamin A in the cell—are obscure even as problems Yet the beginning that has been made with vitamin A metabolism in the study of visual systems seems now to lead in several directions beyond the retina and offers some promise of bringing us to face with these larger issues

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Discussion

Dr Friedman With hesitance I ask if the crystalline vitamin A used by you was the synthetic or natural?

Dr Wald I must confess that I m a little up in the air by now as to what we re buying, but I don t think it would matter very much It is the all trans isomer, but could I ask one of the persons from Distillation Products what is being supplied now?

Dr Harris Whether you got the synthetic all trans" or the natural all trans it would be the same thing

Dr Wald Of course, and I take it that you don t make any distinction in your labelling any longer?

Dr Harris In connection with your revelation that a cis form of vitamin A is specific for the reaction you mentioned, you may expect that D P I will soon have a cis ter preparation for our trans vitamin A to take care of any possible demand

Dr Hickman Well, does not one assume that in the body all the A vitamins eventually produce a balanced mixture from which the body can draw an indefinite amount of the one you wanted?

Dr Wald Well, Dr Hickman, I d like to assume that, but I wonder whether it s true This isomerization is such an easy thing, particularly in a molecule which is not merely being kept at body temperature but going through all kinds of business that you d expect that it would make little difference which isomer one ate Yet, Zechmeister recently has reviewed experiments from his laboratory and Deuel s in which they fed stereoisomers of alpha, beta, and gamma carotene to rats and rabbits and asked the question,

does it make any difference to them as pro vitamin A They found big differences in pro vitamin A potency of the stereoisomeric forms used differences as great as 3 to 1 In only one instance in the rabbit, they found a cis type to work better than the all trans In all other cases the all trans worked better, so that it s quite possible that these molecules preserve their stereoisomeric configuration better than I would have supposed All of this needs to be looked into further, but I think the important thing is that whenever a carotenoid approaches an enzyme from now on, we must look very carefully for effects of configuration, and ask whether the configuration is not one of the determining factors in the reaction

DR WEISBERG I d like to ask Dr Wald if he had time to get any clues as to what kind of protein opsin is—at least what class it belongs to

DR WALD No I m sorry The difficulty in working with opsin—we re doing what we can—but the trouble with the whole business as I ve already told you, but you don t realize how serious it is as yet, is that we can only dissolve opsin in a detergent We therefore, have in our hands never opsin which I think we ve got pretty pure but the opsin detergent complex and this works havoc on all attempts to try to do ordinary protein chemistry with this substance

DR HICKMAN Of course, that s obviously a wise provision of nature Isn t it so that no matter what is ingested the opsin stays insoluble?

DR AMES With reference to Dr Wald s remarks concerning the ability of the rat to handle stereoisomeric forms of carotene, I would like to cite our recently published investigations of neovitamin A When either neo vitamin A alone or trans vitamin A alone was fed to rats, an equilibrium mixture of the two forms consisting of approximately 10 to 15 per cent of the neo form was found in the liver A vitamin A alcohol ester ratio of approximately 1 to 3 was found in the liver irrespective of whether the alcohol or ester form was fed to the animal Apparently the rat can isomerize as well as hydrolyze and esterify the various forms of vitamin A and deposit a characteristic mixture of the various forms in the liver with good efficiency

DR WALD Yes but this is precisely the case in which one wouldn t expect to find much difference I think the problem arises precisely at the point at which some more specific enzyme reaction needs to take place, and there one would have to test as in this opsin case

DR DEUEL We happen to have been the laboratory where all the work was done on the various stereoisomers and I d like to bring up one item discussed in a paper now in press which may have some relationship to what Dr Wald has mentioned We recently received from Dr Inhofen a synthetic mono cis carotene which has the cis linkages at 13 to 15 prime Now Zechmeister in his isolation of products of stereoisomerism of β carotene had

never observed this particular mono cis compound Apparently the reason for that was that this compound is extremely light sensitive and it is imme diately destroyed I don t think it went to the all trans form it was destroyed By taking precautions to keep the material out of the light and by keeping our animals out of the light, we were able to observe a 50 per cent potency of this mono cis β carotene as a pro vitamin A as compared with the all trans form It might be that some of these vitamins A which are so difficult to prepare might also be light sensitive In this particular mono cis carotene, the particular cis peak was the highest of any compounds which have been observed The ratio was 1 to 1 8 between the value at 350 mμ and that at 460 mμ

DR WALD You see now why we have a very strong interest in the cis peak being displayed by rhodopsin itself This matter of stereoisomerization doesn t always require iodine catalysis—it just requires light As long as one has pigment that absorbs the light you re putting in that is enough Vitamin A doesn t absorb much visible light and most ordinary lights don t have much ultraviolet but in ordinary white artificial light retinene is isomerized It takes only a few minutes of irradiation with an ordinary tungsten lamp to isomerize retinene There s an old story in the literature by my friends, Aurin Chase and Emil Smith several years ago which I never believed I never checked it either because I simply didn t believe it It was that if one bleaches rhodopsin with blue light one obtains some regeneration, but if it is bleached with yellow light there is no regeneration Well this is exactly right Blue light not only bleaches rhodopsin but stereoisomerizes the retinene that comes out Yellow light bleaches rhodopsin but since it is not absorbed by the retinene it cannot stereoisomerize it The wrong isomer of retinene comes out of rhodopsin when it bleaches and this must be iso merized to the right isomer before it can re enter the synthesis of rhodopsin

VITAMIN D AND RELATED STEROLS *

CHARLES E BILLS

'HE TERM vitamin D is applied to several antirickétic substances erived from, or associated with, the sterols The existence of the itamin was first indicated in experiments by Mellanby, who found iat rickets in puppies is a deficiency disease, and that cod liver oil ontains a factor which prevents it In 1922 the vitamin was recog- ized as a substance distinct from vitamin A by McCollum, Simmonds, ecker and Shipley The experiment consisted in oxidizing cod ver oil until the antixerophthalmic factor, vitamin A, was destroyed, aving the antirickétic factor which they later termed vitamin D ucker, Pappenheimer and Barnett found that vitamin D appears i the unsaponifiable, or sterol, fraction of cod liver oil, and they uggested that " it may be a sterol related to cholesterol or a holesterol derivative'

Proof of the relationship to sterols followed the discovery in 1924 f the antirickétic activation of foods It was found by Steenbock and y Hess that numerous foods acquire vitamin D activity when xposed to ultraviolet rays Presently it was demonstrated that in oods it is the sterol content which is activated The common sterols f plant and animal fats usually contain as an impurity certain rare terols which are the parent substances of the vitamins D, and are alled the provitamins D

Only two provitamins D of known constitution have been isolated rom natural sources, but others of unknown molecular form occur i the invertebrates, especially mollusks, and still others have been ynthesized

The best known provitamin D is ergosterol, the principal sterol f ergot, yeast and fungi in general In the higher plants ergosterol

* A résumé of the paper read at the Symposium The paper will be published *i extenso* as a chapter entitled Vitamin D in the forthcoming book *The Vitamins* dited by W H Sebrell and R S Harris Academic Press, Inc, New York

appears to be the provitamin D accompanying the major phytosterols It has been identified in grass, wheat germ, cottonseed, and scopolia root It has also been found, along with cholesterol, in animal sources such as earthworms, snails, and hens eggs Ergosterol probably never originates in the animal kingdom, but it is absorbed by certain animals from their vegetable food The mammals, however, apparently do not absorb it

The other naturally occurring provitamin D of known structure is 7 dehydro cholesterol, which differs from ergosterol only in its side chain The relationship is that of a 22 dihydro 24 demethyl ergosterol This is strictly an animal product, never found in the vegetable kingdom It has been isolated only from pigskin, whelks, and ducks eggs, but indirect evidence from chick rat vitamin D assays of irradiated foods indicates that it occurs widely in animal products, as does ergosterol in plant products

Provitamins D of unknown structure occur abundantly in the unsaponifiable matter of the fat of invertebrates They probably possess the same ring structure as the other provitamins, but have somewhat longer and otherwise different side chains

Several provitamins D have been prepared from the corresponding common sterols by semi synthesis For this purpose, three types of reactions have been developed (1) dehydrogenation of common sterols at C 7, (2) dehydration of 7 hydroxy sterols, and (3) dehydrobromination of sterols selectively brominated at C 7 A few provitamins have been made by reactions of less general applicability

The structural features which differentiate the common sterols, provitamins D and vitamins D are shown in the figures below The ordinary sterols, such as cholesterol and sitosterol, have one double bond in the ring system, always at C 5 The provitamins D have a second double bond at C 7 The vitamins D have these same two double bonds, plus a third one at C 10, which results from a rupture of ring B during activation

The energy required to rupture the ring and form vitamin D is usually supplied by ultraviolet light, but activation can be brought about, albeit less efficiently, by electrical discharges, cathode rays or radium emanation When ultraviolet light is used, about $9\ 3 \times 10^{13}$

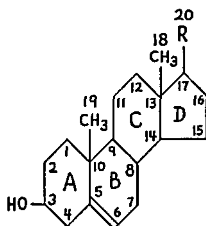


FIG 1 Common sterols

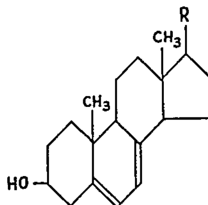


FIG 2 Provitamins D

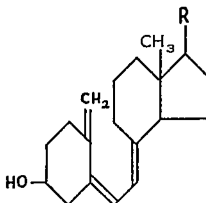


FIG 3 Vitamins D

quanta are required to produce one I U of vitamin D_2, and the efficiency is largely independent of the wavelength

The provitamins D, because of their conjugated double bond system, exhibit a characteristic absorption spectrum of four maxima in the ultraviolet Even great differences in the structure of the side chain, R, make scarcely any difference in the position of these maxima When exposed in solution to ultraviolet light, the pro vitamins D undergo a series of spectral changes corresponding to the formation of a series of irradiation products in overlapping steps Regardless of the structure of R, the spectral changes are essentially identical, which indicates that analogous products are being formed in all cases at the same stages of irradiation This conclusion is supported by the isolation and analysis of a number of the inter mediates In short, when the R analogs of ergosterol are irradiated, the corresponding analogs of calciferol are produced

The animal body is keenly sensitive to structural differences in the side chain or in other parts of the vitamin D molecule, so that some of the vitamin D analogs are highly antirickctic, some moderately so, and others seem to be devoid of healing action There are marked differences also in the response of different species to a given form of the vitamin Thus it is that vitamins D_2 and D_3 are equally effective, per microgram, for rats and probably for all mammals, yet D_3 is 100 times as effective as D_2 for chickens and other birds Unfortunately the testing of the vitamin D analogs for antirickctic action has often been limited to rats and to minute doses given orally A great field remains unexplored, in which various species, widely different doses and both oral and parenteral administrations should be used to answer the poser, when is a vitamin not a vitamin?"

Calciferol, or vitamin D (the Viosterol of commerce), comes from ergosterol, where $R = CH(CH_3)\ CH{=}CH\ CH(CH_3)\ CH(CH_3)_2$ It is highly antirickctic for mammals, relatively ineffective for birds Vitamin D_3 comes from 7 dehydro-cholesterol, where $R = CH(CH_3)\ CH_2\ CH_2\ CH_2\ CH(CH_3)_2$ It is highly effective for mammals and birds Vitamin D_4 comes from 22 dihydro ergo sterol, where $R = CH(CH_3)\ CH_2\ CH_2\ CH(CH_3)\ CH(CH_3)_2$ Its side chain is saturated as in vitamin D_3, but contains the additional

methyl group of vitamin D_2 Its antirickctic properties are diminished and its chick rat efficacy ratio is intermediate From 7 dehydro-campesterol there is obtained a vitamin D which is a side chain epimer of vitamin D_4 It is less potent than D_4 for rats, being about one tenth as active as D_2 When the double bond in the side chain of vitamin D is saturated with an oxido group, the resulting oxido calciferol exhibits a sharply reduced potency A highly potent iodo calciferol, and a feebly active calciferol ketone have been prepared Vitamin D esters are active only if they are hydrolyzable by the animal body Epi calciferol and epi vitamin D_3, which differ from the normal vitamins in the steric arrangement of the hydroxyl group, are moderately active The vitamins D obtained from the mollusk provitamins of undetermined side chain structure resemble vitamin D_3 in tests with animals, but some of them may have an even higher chick rat efficacy ratio

The most inadequately tested vitamins D include the following The vitamin D analog from 7 dehydro stigmasterol, where R $= CH(CH_3)\ CH = CH\ CH(C_2H_5)\ CH(CH_3)_2$, seems devoid of activity This product differs from calciferol only in having an ethyl group in place of a methyl group in the side chain The vitamin D analog from 7 dehydro sitosterol, where R $- CH(CH_3)\ CH_2\ CH_2$ $CH(C_2H_5)\ CH(CH_3)_2$, was once thought to be active, but its activity appears to be due to impurities This product differs from vitamin D_4 only in having an ethyl group in place of a methyl group in the side chain The ability of the animal organism to distinguish ethyl from methyl in the side chain of a massive molecule is remarkable 7 Dehydro clionasterol, a side chain epimer of 7 dehydro sitosterol, has been synthesized but has not been tested for activatability Other sterols of the 7 dehydro, or provitamin D, class are known, but little has been learned about the corresponding vitamin D analogs When the hydroxyl group of vitamin D_3 is replaced by chlorine, bromine or mercaptan, the resulting compounds are not antirickctic The hydroxyl group of provitamin D_3 has been replaced by a hydroxymethyl group, but no studies on the activatability of the resulting compound have been reported

The richest natural source of vitamin D is fish oils, especially

fish liver oils, in which it occurs largely as esters with fatty acids The predominant form is vitamin D_3, but calciferol has also been identified in tuna liver oil Evidence from molecular distillations indicates the presence in cod liver oil of two major, two minor and two trace forms, but the structure of none of these has been determined Some of the vitamin D of fish oils may originate by the insolation of plankton, especially the common seaweed, *Sargassum* However, the vitamin D_3, according to current theory, must be of animal origin, and the most plausible explanation of its predominance is that it originates by synthesis in the fish and without the agency of light

For the estimation of the duration of the rickets we relied chiefly on the extent to which endochondral ossification was affected If growth is preserved, given time enough, severe and moderate rickets will leave characteristic patterns at the end of the bone, consisting of typically deformed proliferative cartilage and trabeculae If this rachitic zone was broad, for example 1 cm or more at the anterior end of one of the middle ribs, we were able to infer that the disease had been present for some time and could classify the case as chronic At the opposite extreme, if the rachitic disturbance consisted merely in defects in calcification of the provisional zone of the cartilage with or without beginning invasion and there was no rachitic zone at all, meaning that not enough time had elapsed since the beginning of the disease for the end of the shaft to develop the defects in pattern, we classified the case as acute, except in numerous cases of slight rickets in which we could not exclude the possibility that the disease might have been subacute or chronic, as will be explained later The classification of subacute was made when evidence for the disease was present but was not extensive enough to indicate long duration We hasten to point out that the division of our cases in terms of duration was subject to great difficulties of interpretation and some error on the side of omission As is well known, the rate of growth at the different ends of the long bones of the body varies tremendously It is most rapid in the middle ribs, next at the lower end of the femur and slowest at the upper ends of the radius and ulna, particularly the latter Severe chronic rickets in one of the middle ribs might be evidenced by a zone a centimeter in length whereas at the upper end of the ulna the lesion would be limited to the cartilage shaft junction From the examination of the upper end of the ulna one might infer that the rickets had been of extremely short duration and from the rib that it had been chronic We easily got around the difficulty of the variation of the growth rate in the different ends of the long bones because in almost every case one of the middle ribs was available for study It was on the findings in one of the middle ribs that we depended chiefly for our estimation not only of the duration of the disease but also its degree But a difficulty which we could not circumvent was that the general

growth rate must have been subject to great variation in our series of children, since they were not only of different ages but died from a whole variety of diseases of different severities and durations which doubtless had different effects on growth There was no means by which we could estimate the extent to which the growth rate had been affected by the disease, nor are there accurate data concerning the increments of growth at the different bone ends at the different months of infancy In many cases there was evidence that growth had been subject to checks, in some it had obviously been greatly reduced and in a large number, in particular the subjects of acute, violent infections like dysentery, growth had virtually ceased In general, however, it was remarkable to see the evidence afforded by the rachitic lesions in the bones that growth had persisted in spite of prolonged serious illness We were aided in other ways in making judgments of the duration of the disease For example, if the baby was young enough, the disease was obviously acute In some cases histological sections of the upper ends of the radius and ulna and in a considerably larger number X ray films of those bone ends were available If the rickets could be well established by either method at the upper ends of the radius and ulna, the disease was obviously chronic We could tell that the rickets was chronic, also, in cases in which the cartilage had accumulated in large mass at the end of the shaft and the invading vessels, those entering from the cartilage side the cartilage canals, and those ascending from the shaft side had time enough to become large bush like structures At times we were aided in estimating that the duration of the disease had been long because of the wideness of the osteoid borders of the trabeculae Extremely broad borders indicated chronicity But the development of the osteoid borders was subject to great variation and in a good many cases in which the rachitic lesion at the end of the bone was extensive the osteoid borders in the shaft were not particularly broad and in some were almost nonexistent We were often aided in the recognition that the rickets should fall into the subacute class because the vessels entering the cartilage from the shaft had succeeded in penetrating too far for acute rickets In yet other cases we could see that the disease must have been at least

subacute because of the depth which the proliferative cartilage had attained and also because of compression deformities of the cartilage at the shaft junction

We encountered equal difficulties in estimating the degree of the rickets Our chief criterion for estimating the degree was the extent to which the deposition of inorganic material appeared to have been affected at the cartilage shaft junction We knew, of course, that the rickets was severe when lime salt deposition in the cartilage was entirely suspended But this state of complete rickets was encountered only occasionally and then only in cases in which the disease was acute or subacute In almost all instances of chronic rickets, scattered deposits of inorganic salts which from their pattern could be identified as having occurred in cartilage matrix could be found somewhere in the rachitic area Balance studies in rickets in the human being have repeatedly shown that negative balances almost never occur, i e that some deposition of calcium, phosphate and carbonate must be occurring somewhere in the skeleton even when the rachitic state is most severe, it is noteworthy in our series that in no case in which the rickets had evidently been in progress for a long time were localized inorganic depositions entirely wanting If the deposition of the lime salts in the middle rib was severely inhibited so that only a few scattered depositions could be found in microscopic examination, we called the rickets severe On the other hand, if the provisional zone of calcification of the cartilage showed normal calcification everywhere except in a few isolated points, we classified the case as slight We classified cases as moderate when deposition of inorganic materials was considerable but irregular with areas of deposit alternating or intermingled with areas which had been completely spared In estimating the degree of rickets we did not get much help from the osteoid on account of its variability resulting probably from general nutritional conditions rather than from the effects of the rickets itself We did of course obtain help from the examination of osteoid in the identification of healing and healed rickets

Our classification gave rise to nine combinations but two of these proved impractical We were able in some children to be certain that slight rickets was acute, but in many cases it was impossible to be

sure that it might not have been subacute or chronic, in other words, we could not recognize subacute or chronic rickets which had remained slight The reason for this was that rickets, when slightly developed at the cartilage shaft junction, that is when limited to a few spotty defects in calcification of the provisional zone, does not leave behind it any record in the form of deformed trabeculae in the shaft distinct enough for recognition This finding of ours is important in the evaluation of the only study of the incidence of rickets similar to ours, namely that of Schmorl which will be discussed below

Subacute moderate rickets was our dumping ground Its cases exhibited a wide latitude both in duration and degree, for, if we could not classify a case as definitely chronic and severe or slight, we put it in the subacute category

When healing occurred in rickets which had reached either the subacute or chronic stage, it was easy to recognize from the presence of a telltale sheet of inorganic materials, always incomplete, stretching across the cartilage at the level of the provisional zone of cells It is important to emphasize that if this sheet occupied the level of the provisional zone, we knew that healing was occurring at the time of death In many cases, however, such sheets lay in the cartilage on the shaft side of the provisional zone of calcification When this occurred it was possible to infer that a healing episode had occurred but the rickets had broken out again subsequently and was severe at the time of death We were aided also in recognizing that active healing was in progress when the osteoid borders showed calcification along their junctions with the prerachitic calcified bone

We were able to recognize healed rickets when we found that calcium phosphate deposition in the cartilage had become fully resumed and the trabeculae normally calcified In many cases of healed rickets indubitable evidence that it had been moderate or severe lay in the scars left by the disease which consisted not only of deformed trabeculae but often in addition of islands or strands of degenerated cartilage which had not yet been converted into bone and removed

We have called attention to various difficulties encountered in

estimating the duration and the degree of the rickets But perhaps the greatest difficulty of all in classifying our cases lay in the fact that in many instances the disease at the time of death was not stationary but was rapidly changing its severity For example, the study of the rachitic area might show that the disease had been of moderate severity but at the time of death had become so severe that deposition of calcium phosphate had stopped altogether In other instances the movement of the disease had occurred in the opposite direction, for example, a rickets which had been clearly severe or moderate had begun to show signs of healing In still other cases there was evidence that the disease had fluctuated to and fro

In experimental rickets in the rat the problem of estimating the degree of the disease is simple since the animals are standardized in regard to age or weight and controlled In the case of our children, as already pointed out, no standardization was possible because the ages were different and because of disease factors Our criteria of rickets could not be based on fixed measuring scales of any kind In consequence our judgments on how to classify our cases were entirely subjective and resolved themselves into decisions based on whatever evidences the individual cases offered in the light of our experience It is obvious that our classification is subject to great error and of the roughest sort but we believe that it will serve the purpose of giving correct general ideas adequate to meet the clinical requirements

I shall first discuss the prevalence and severity of rickets in the group of 1,303 children aged from birth to the end of the second year As will be seen two incidences of the disease were detected during the first two weeks in babies aged 12 and 14 days respectively These were classified as acute slight No case of congenital rickets was found in a series of 162 stillborn infants From the first month on the prevalence rises and reaches a peak of 91 per cent during the seventh month, falling thereafter, so that during the second year the prevalence for the 177 subjects studied was 63 per cent In Table 1 there is a breakdown of the cases in each age period into slight, moderate or severe rickets Rickets of slight degree comprised

one quarter to one third of the total, i e rickets varied from moderate to severe in three quarters to two thirds of all cases

In Baltimore the negro population comprises about 20 per cent of the total In a given year discharges from the Harriet Lane Home of negro children under 2 years of age average about 50 per cent of the total However, a factor which produces an increase in the

TABLE 1

PREVALENCE AND SEVERITY OF RICKETS IN A CONSECUTIVE SERIES OF 1,303 AUTOPSIES ON CHILDREN AGED FROM BIRTH TO THE END OF THE SECOND YEAR

Age	No Subjects Examined	No with Rickets	Percent with Rickets	Percent Slight	Percent Moderate	Percent Severe
0 15 das	360	2				
16 30 das	90	55	61 2	36	49	15
1 mo	99	48	48 5	21	67	12
2 mo	90	69	75 9	9	48	43
3 mo	69	57	82 7	19	63	18
4 mo	79	62	78 5	39	29	37
5 mo	73	56	76 8	30	52	18
6 mo	53	44	83 0	21	52	27
7 mo	54	49	90 7	25	47	28
8 mo	40	30	75 0	23	63	14
9 mo	38	30	79 0	27	43	30
10 mo	41	26	63 5	30	35	35
11 mo	40	24	60 0	21	37	42
12 23 mos	177	113	63 8	38	30	34

negro percentage in our series of cases is that the percentage of autopsy permissions is higher in the negro group In Table 2 are the data on the prevalence of rickets in white and negro children in the age group from birth to the end of the second year The percentage of cases of rickets in the white group is a little lower than in the negro group From the first month through the eleventh month the incidence is 68 per cent for the white and 72 per cent for the negro children The disease tends to be more severe in the negro as shown by the fact that the percentage of cases classified as moderate and severe is greater in the negro children Moreover, if only the cases designated as severe are considered, there were 21 or 11 8 per cent among the 178 rachitic white children and 115 or

36 3 per cent among the 317 negro children autopsied Similarly in the second year the prevalence and severity of the rickets are greater in the negro than in the white group, for example 45 5 per cent for the white and 72 2 per cent for the negro Similarly if only those cases considered moderate or severe are considered, the per centage among the negro children was 69 6 per cent and among the

TABLE 2

Prevalence and Severity of Rickets by Color in the Group of Children of Table 1

Age	No White Subjects	No with Rickets	% with Rickets	% Moderate and Severe Rickets	No Negro Subjects	No White Subjects	% with Rickets	% Moderate and Severe Rickets
0 15 days	174				186			
16 30 days	41	25	60 9	61 0	49	30	61 4	70 0
1 mo	48	19	39 5	68 5	51	29	56 9	86 2
2 mo	34	21	61 8	90 5	56	48	85 8	91 7
3 mo	31	25	80 5	72 0	38	32	84 3	87 5
4 mo	26	22	84 6	41 0	50	40	75 6	80 0
5 mo	25	17	68 0	70 5	48	39	81 3	69 3
6 mo	19	15	79 0	60 0	39	29	85 3	89 7
7 mo	22	19	86 4	63 0	32	30	93 8	83 3
8 mo	18	13	72 2	77 0	22	17	77 3	76 4
9 mo	12	10	83 3	70 0	26	20	22 0	75 0
10 mo	16	11	68 9	54 5	25	15	60 0	80 0
11 mo	12	6	50 0	33 3	28	18	64 3	94 3
12 23 mos	68	31	45 5	45 1	109	82	75 2	69 6

white 45 1 per cent Only 4 out of 68 white children, 5 9 per cent, in comparison to 34 out of 109 negro children or 31 2 per cent showed the disease in severe form

All clinicians know that the premature infant is far more prone to develop rickets than the infant born at term and side by side with the latter will develop the disease in severer form All I shall say in regard to the relationship of prematurity to the presence of rickets in this group of infants is that the analysis of our data failed to show a greater prevalence or greater severity among the premature infants We have no explanation to offer for this divergence of our data from accepted knowledge other than that the premature infant is more carefully nurtured and the mother under more frequent

contact with doctors and nurses than the more robust infant born at term Theoretically it would seem possible that protective measures through the use of vitamin D might have been applied more closely in the case of the premature infants than those born at term but an analysis of our data with this possibility in mind still failed to demonstrate that this factor was the cause Our data also failed to reveal seasonal incidence of the disease although the existence of a seasonal prevalence in the winter and spring months is also an established clinical fact

TABLE 3

INCIDENCE OF RICKETS IN CHILDREN AGED FROM 2 TO 14 YEARS ARRANGED IN TRIENNIUMS

The group was composed of 230 children examined in consecutive autopsies

Rickets After Second Year
Incidence in Trienniums from 2nd to 14th Years

Age yrs	Total	Rickets	Per cent
2 5	132	67	50
5 8	48	19	39 5
8 11	32	14	43 7
11 14	18	7	38 8

I now turn to the analysis of our data concerning incidence of rickets in the age group 2 to 14 years inclusive comprising 230 children The total prevalence of rickets was found to be 46 5 per cent The figures according to years indicate that the disease occurred with scarcely diminished frequency up to the fourteenth year In 23 per cent the disease was classified as slight, in 18 7 as moderate and 4 8 per cent as advanced In the white children the total prevalence was 43 6 per cent and in the negro children 48 5 per cent However, the prevalence of moderate and of advanced rickets taken together was 27 per cent in the negro as compared to 18 per cent in the white children and advanced rickets was limited exclusively to the negro children Thus although the total prevalence of rickets was not greater in negro children, the prevalence of the disease in well developed degree was greater among them than among the white children

Our studies of the incidence of rickets in this older group of children indicated a definite seasonal variation, the greatest prevalence was in the winter months, the lowest in the autumn months

Before leaving the subject of the age incidence in the older group of children it is advisable to point out that the disease was insufficiently developed to show clinically in the great majority of cases and, furthermore, that it was also limited to manifestations in the shaft which could not possibly be recognized either clinically or by

TABLE 4

PREVALENCE AND TYPE OF RICKETS IN SCHMORL S SERIES FROM 2 TO 48 MONTHS OF AGE COMPILED IN DRESDEN FROM CONSECUTIVE AUTOPSIES BETWEEN 1901 AND 1908

As explained in the text Schmorl s study embraced 386 children aged from 2 months to 4 years Schmorl s data in the table below after the twenty fourth month have been omitted The object in the omission was to make Schmorl s age period correspond to ours so that his data could be compared directly to ours

Age Mos	Beginning		Florid		Healing		Healed	
		%		%		%		%
2	4	100						
3	12	75	4	25				
4 6	19	57 6	7	21 2	7	21 2		
7 9	17	34	23	46	10	20 0		
10 12	7	9 5	45	61 7	20	27 4	1	1 4
13 18	4	6 9	32	55 2	15	25 8	7	2 1
19 24	2	6 6	10	33 4	11	36 6	7	23 4
25 36			13	22 9	14	24 5	30	52 6
37-48			2	8 3	3	12 5	19	79 2

X ray in the great majority of cases In children aged from 2 to 3 years the disease appeared in the shaft alone in 21 cases, in the cartilage shaft junction in 4 cases and in both in 13 cases But as the child grew older the occurrence at the cartilage shaft junction became less and less common In only six cases, all told, was it recognizable in the X ray film

I now turn to the study of Schmorl, which, as already stated was based on the study of 386 children aged from 2 months to 4 years autopsied in Dresden, Germany, between 1901 and 1908 But before giving the incidence of rickets reported by Schmorl it is necessary to make clear differences in his approach to the problem from ours

First he used a different classification Schmorl divided his cases into beginning florid healing and healed rickets Beginning rickets occurred when the disease was demonstrable microscopically but did not show macroscopically or else was not evident enough on examination in the gross to make its presence certain Rickets was florid when the disease was recognizable macroscopically but healing was excluded by microscopic examination Healing rickets was characterized by the presence of newly deposited inorganic material in the provisional zone of the proliferative cartilage as identified by microscopic examination Healed rickets was recognized when either characteristic rachitic gross deformities in the contour of the bone or rachitic deformities of the finer structure as revealed by the microscope were present but the osteoid did not exceed normal thickness and the cartilage failed to show fresh evidences of rickets though it might still show deformity Another difference in Schmorl s approach to the problem was that as the result of preliminary studies he assumed rickets did not occur in the first month of life and for that reason he stopped examining infants for rickets during the first month so that that period was left out altogether

Although Schmorl s study embraced 386 children aged from 2 months to 4 years we have tabulated only those portions of data which applied to children aged from 2 months to 24 months in order to bring his figures into direct comparison with ours It will be seen from an examination of the table that rickets began in the second month and from the second to the sixth month beginning cases prevailed and were common from the seventh to the ninth month From the tenth month on beginning rickets gradually decreased although cases were encountered to the end of the second year Florid rickets was rare to the sixth month but became frequent from the seventh month on dominated from the seventh to the ninth month and reached its maximum at the end of the first year From the thirteenth month on it decreased Healing rickets appeared in the second quarter of the first year and in the last half of the second year rose to 56 6 per cent Healed rickets was not encountered until the second year The cases increased from a

minimum of 1 to 2 per cent in the first half of the second year to 23 per cent in the last half

Comparisons of Schmorl's results with ours are difficult One reason is that his criteria of rickets differed so widely from ours He depended largely on examination of bones in the gross We found gross examination as a means of detecting rickets most unreliable, when checked by microscopic examination, except when the disease was far advanced We could not recognize ' beginning ' rickets with enough certainty to make a separate classification of it for the following reasons ' Beginning " rickets is slight rickets Slight rickets, as already pointed out, does not leave behind it any mark in the shaft, consisting in deformed trabeculae Although often we could be sure, particularly from the age, that slight rickets was " beginning " rickets, in many cases we could not be certain that slight rickets was not subacute or even chronic All clinicians are aware that rickets in slight as well as severe form may be present for many months We think that Schmorl's designation of " beginning " rickets should be changed to slight rickets, a classification which does not make any commitment as regards duration Schmorl's " florid rickets should correspond to our chronic and subacute severe and moderate groups combined, probably severe more than moderate A reason for thinking this is that Schmorl's " florid ' rickets was apparently unattended by healing in the form of inorganic salt deposition But, as already pointed out, in our experience, the discovery of either subacute or chronic rickets without scattered foci of inorganic deposition either in the proliferative cartilage or in the metaphysis was a great rarity, although we did find cases of acute and subacute severe rickets in which fresh depositions of calcium phosphate everywhere were lacking We were completely unable to make a satisfactory separation of " healing " rickets although in some of our cases obviously fresh healing was certain since deposits of blue staining material were present in the provisional zone of the proliferative cartilage, in many other cases there was evidence that the healing had been temporary and the rickets had started up again In other words our healing group was too subject to error in interpretation to be included as an entity If we had classified healed rickets

as a separate group our cases would have been scattered throughout the series

Every clinician knows that beginning rickets would be most com monly encountered in the early months, that chronic and subacute severe rickets would predominate in the middle period and that toward the end of the second year healing and healed rickets would be found with increasing frequency Schmorl's data came out exactly as the clinician expects from his experience with rickets and exactly as the clinicians found in the Harriet Lane Home in the early years before rickets was treated and prevented by means of vitamin D Possible reasons why our data do not show this orderly sequence in the development of the disease and Schmorl s data do show it are as follows The latitude of Dresden is 51° whereas the latitude of Baltimore is 39° Consequently the rachitic period, that is the period when the ultra violet radiations of the sun which are effective against rickets fail to reach the surface of the earth, is considerably longer in Dresden than in Baltimore Thus one would expect that, all other conditions being equal, the prevalence of rickets and also the severity would be greater in Dresden than in Baltimore Moreover the climate in Dresden is much colder in the winter period and the winter period itself is much more prolonged in Dresden than in Baltimore so that the tendency to stay indoors and hidden away from any existing antirachitic radiations would be greater in Dresden than in Baltimore The discovery that cod liver oil was effective in rickets was made by Mellanby (3) in his experiments on dogs in 1919 and in the same year Huldschinsky (2) reported the curative action of ultra violet light in rickets Thus the therapeutic period by which rickets could be prevented or cured through exposure to sunlight or treatment with cod liver oil began shortly after 1919 Schmorl's studies were made before these discoveries were known in the period of therapeutic nihilism so far as rickets was concerned In conse- quence Schmorl probably encountered rickets in its simon pure form and he was able to demonstrate a sequence in the manifestations of the disease In contrast the studies by Follis, Jackson and Park (1) and also our series of children under 3 years, not yet reported, were made after 1919 when the enthusiasm in regard to the prevention of

rickets both by treatment with vitamin D and exposure to sunlight and also to artificial sources of anti rachitic radiations was at its very height There was indeed a special clinic in the Harriet Lane Home devoted to the prevention and cure of rickets We believe, first, that the prevalence and also the severity of rickets in Baltimore was never so great as the prevalence and severity of the disease in Dresden This opinion is based entirely on theoretical grounds, resting on the more northerly latitude of Dresden But we believe also that our statistics were thrown awry, so that they did not show the sequence noted by Schmorl and the natural prevalence and severity of the disease because of the employment of vitamin D Our investigations of the clinical histories indicate that in some cases vitamin D was not given at all, in other instances that it was given irregularly and in many instances that it was given for a period or periods and then stopped Thus the normal course of rickets was prevented from becoming clearly manifest

Probably the most important result of our studies, which are far more extensive than those of Schmorl, not only in numbers of cases but also in age range examined, is the demonstration that in spite of the knowledge of the preventive action of vitamin D and also of ultra violet light, rickets remains in the Baltimore community an extremely common disease, in all probability the commonest of all diseases to which children are subject, and that knowledge which is sufficient to abolish the disease entirely from the community has failed to accomplish this

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DISCUSSION

DR PAPPENHEIMER I wish to ask Dr Park if he thinks it possible that Maxwell s case may have been an example of low calcium rickets I saw those slides back in 1931 and it seemed to me at the time that the picture as you showed it on the screen was not quite that of the usual type of rickets There was no excess cartilage Notoriously of course the Chinese diet is low in calcium

DR PARK I think that may very well be the case and it certainly is typical I bring out another point the evidence is rickets being limited to the cartilage shaft junction and no osteoid showing itself Dr Follis and I have been tremendously impressed with the fact in our studies that the growth of the bone in length that is, the growth of the cartilage and the new formation of bone on the surfaces of the trabeculae are under different regulations and that one may go on and the other stop It s been in very severely sick children an exceedingly common thing for us to find sometimes very marked rickets at the end of the bone and no osteoid at all and that is born out by the fact that the osteoblasts on bone surfaces have become few and spindle shaped and yet a very considerable degree of growth has taken place at the end of the bone So in our cases we could give many examples in which the rickets declares itself in very marked form at the end of the bone but the osteoid is entirely lacking

DR V E LEVINE What would be the effect of other vitamin deficiencies on the histological bone picture? What would be the effect of an excess of vitamin D intake?

DR PARK An excess of vitamin?

DR V E LEVINE These children may have had other deficiencies besides vitamin D deficiencies or they may have had an excess of vitamin D intake Extensive changes have been reported in the bones in vitamin A and in vitamin C deficiency

DR PARK Well in regard to excess of vitamin D intake I shall refer you to Dr Follis because Dr Follis has just concluded a study of that very thing I don t know of any other vitamin deficiency than that of vitamin D which produces disturbances in the calcium phosphate metabolism Vitamin A deficiency would produce disturbances in growth but it produces no dis turbances in the calcium phosphate metabolism so that no evidences of rickets show themselves Vitamin C deficiency scurvy of course produces a very well understood picture but there s no evidence that it disturbs the calcium phosphate metabolism and it never produces a picture which resembles rickets in any way In fact the vitamin C deficiency works in the direction of preventing the rachitic lesion from developing As far as the B complex is concerned I don t think there s any evidence there that deficiencies inter fere in any respect with calcium phosphate metabolism and consequently that they never are the cause of the development of rickets

DR SOBEL May I add a few comments and then wind up with a question First there may be an interesting explanation for the lack of incidence of rickets at birth namely Robison has shown that when an embryonic bone is calcified *in vitro* the Ca × P product required for new calcification is only 16 as contrasted to a Ca × P product of about 35 for the usual type of rachitic bone which will not mineralize at a Ca × P product of 16 The findings

suggest that the calcifying mechanism in the embryonic bone matrix is so rich that at calcium and phosphate levels *in the body fluids at which* older bones will not calcify, there will be still avid uptake of lime salts, and possibly this is the reason why at birth we do not see rickets

As for the second question, why is it that in infectious diseases such a high incidence of rickets was observed, there is an interesting analogy between vitamin A deficiency reported by Blackfan and Wolbach *in 1933 in children* with pneumonia, fed large doses of vitamin A, and the observations of Dr Park Later work seems to imply that in such diseases the absorption of fat *and fat soluble vitamins is impaired even more than is usually the case for* the young child, and all evidence to date from our studies and those of others indicate that children under three to six months of age absorb fat very poorly *and absorb fat soluble* vitamins in oil equally poorly compared to older children There are exceptions about 30 per cent of young children don't belong in this category, 70 per cent seem to belong in this category, and that 70 per cent may not do well when given vitamin D in low potency oil With highly concentrated vitamin D, the problem of dispersing it in the intestine is therefore relatively simple I don t know what the facts are in Baltimore, but if the prescription is that of cod liver oil, which is a more dilute source of vitamin D, the problem of adequate intestinal dispersion by the bile acids and other agents is more difficult and, therefore, the likelihood of a deficiency on adequate intake increases

While it is recognized that poor intestinal absorption can cause a frank avitaminosis, it is not generally recognized that similar vitamin deficiencies can be the result of the breakdown of the transportation system to the site of action Theoretically, it is evident that if the membranes of the blood capillaries or the lymph were completely impervious to the passing of fat soluble vitamins there should be deficiencies in the tissues requiring the vitamin That such a possibility actually exists is hinted at by a wide variety of skin diseases, in which keratosis exists resembling vitamin A deficiency, and which respond only to huge amounts of this vitamin Avitaminosis reported by Blackfan and Wolbach in 1933, and the rickets reported by Park and his colleagues, on adequate intake, might fall into the same category That this is a real possibility can be surmised from two observations In a wide variety of febrile conditions, the serum vitamin A level falls, in many cases reaching zero levels In the study of transfer of vitamin A to milk it was *indicated that the critical factor is the serum level* The higher the serum level, the greater the degree of transfer Extrapolating this to lower levels, one would reach the conclusion that there is a serum level at which transfer is almost nonexistent or exists to a low degree If making the most favorable postulate, that vitamin A transfer exists across the capillaries and tissue cell walls by a chemical mechanism independent of serum levels, it certainly could be visualized that this mechanism can break down in a manner analogous to the mechanism of gastrointestinal absorption

One can readily admit that blood levels will not be the only factor in transfer For example, whether the vitamin is ultrafiltrable or not will be a factor Evidence on ultrafiltrability of the fat soluble vitamins is practically nonexistent, except for the studies of Klopp, *et al* (unpublished), who have shown that when the serum level of the vitamin reaches approximately 400 I U per 100 ml, the vitamin becomes ultrafiltrable The conditions that result in ultrafiltrability merit study for the further understanding of vitamin transportation

As far as infectious diseases are concerned, we observed many years ago the same thing that Harrison has observed, for example, in pneumonia inorganic phosphate drops to an extremely low level Naturally, we know that if one takes a bone and tries to calcify it *in vitro* at a low Ca × P product, there will be no new mineralization Alternately, due to poor intestinal absorption, there may be a second phenomena It is possible that the low serum phosphate is due to the vitamin D deficiency, but it is also possible that the disease, by producing a low Ca × P product, produces the equivalent of a vitamin D deficiency The question that I meant to ask you was what is the source of vitamin D in Baltimore that was administered to these children?

DR PARK At the time that these studies were made, it was cod liver oil viosterol

DR KRAMER Dr Park, the terms hypertrophic and atrophic rickets are often used in the literature Do you consider these as having any significance?

DR PARK It s an X ray term, isn t it? If the bone has become a robust bone, it s spoken of as hypertrophic, or, on the other hand, if in the X ray it shows as a very much rarefied rachitic bone, it s spoken of as atrophic Oftentimes in the so-called hypertrophic rickets one finds tremendously wide osteoid borders and, obviously, a great many children with rickets grow very well and they become great big children and they have big bones, and I think the bone really appears bigger in the X ray and wider than it would be normally because nature having to use poor material puts in a lot of it to compensate by quantity for quality The atrophic rickets almost always occurs in very malnourished children and I presume that they are children in whom the destruction of the old bone has been very severe, and I think they are cases in which probably the organism has been obliged to draw on the calcium phosphage deposits in the old bone which has been destroyed in very large part which makes the shadow appear so rarefied

DR PAPPENHEIMER Were the children who had this low inorganic phosphate during the infection receiving ordinary amounts of cod liver oil?

DR PARK Ask Dr Harrison that He s here Dr Harrison—

DR HARRISON Yes, they were During the course of the infection they were usually receiving a constant supplement of vitamin D which had no

effect on the serum phosphorus values until the infection was cleared up and then the serum phosphorus would rise rapidly to normal levels

DR PAPPENHEIMER The reason I asked that question was that many years ago before we had chemotherapy, Gerstenberger reported that with a drop in the serum phosphorus during pneumonias and following crises, the inorganic phosphorus spontaneously rose

DR FOLLIS In the cases of acute diseases that you studied, Dr Harrison, might not it be possible that vitamin D was given insufficiently long enough to produce any effects?

DR HARRISON In some of the patients the acute stage of the disease lasted only 4 or 5 days Of course, the maintenance doses of vitamin D given would hardly be effective in that time In other patients however, the active suppurative injection lasted for several weeks and, despite the administration of vitamin D during that time there was no persistent rise of the serum phosphorus levels until the infection was cleared up There seems to be some effect of an active bacterial infection on the concentrations of serum phosphorus which is quite striking

DR FOLLIS In regard to the question of the excess of vitamin D there is such a thing as hypervitaminous rickito which has been recognized for a number of years It is, of course, rather paradoxical especially so when large quantities of vitamin D will raise the serum calcium and phosphorus in man and other animals by several milligrams per cent We've been studying this question recently and it s not ordinary rickets in that there are no changes of cartilage shaft function of normal rats given as much as 35,000 units of vitamin D a day but there is a great quantity found in the shaft after a week of osteoid and it seems peculiar that that osteoid does not calcify in the presence of supernormal levels of calcium phosphorus in the serum We've been able to calcify that osteoid by normal serum so that there is some peculiarity present here

DR PARK Dr Kramer, I might add that Dr Follis and Miss Jackson and I have attempted to correlate rickets with diseases and we've failed entirely to find any relationship of any particular diease to rickets But I might say that we have numerous examples of rickets which have occurred in children suffering from leukemia and in some from lead poisoning Our series is in both instances too small to have any statistical verification I think that there were 18 cases of leukemia In lead poisoning the explanation might be that the lead combined with the phosphorous and produced a phos phorus deficiency but why it should occur in leukemia seems very queer There were 18 cases, Dr Follis, and half of them showed evidence of leukemia?

DR FOLLIS Yes

DR PARK Half of them showed evidence of rickets and a number of them were older children, 8 to 10 and 12 years of age when rickets might not be expected It s a very curious thing

DR KOHMAN In the case of the older children—is there any evidence as to how long they ve had rickets? Had they acquired it early in childhood or was it a likelihood of acquiring it in later years at an age of as much as 8 years?

DR PARK I don t think I could answer that question very well If rickets is very severe in childhood it produces characteristic bends of the bones which mark the disease as having originated in childhood as long as the individual lives and among the Negroes in Baltimore it is very common to see a man or a woman walking along the street with deformities of such a nature The bends occur in such places that we know that we can date the disease back to childhood In these cases in which the disease occurred in older children there was no evidence that it had dated back to an early period The changes weren t very different from those which Dr Follis has found in cases of chronic nephritis in the bone The rickets couldn t show itself in the X ray picture since it consisted in the majority of cases in just an excess of osteoid substance in the bone itself and there would be no way in which we could be able to say how long the rachitic condition had been there

DR KRAMER Dr Park would you say that your results indicate that the amount of vitamin D that we are using now as a prophylactic is inadequate?

DR PARK I don t know I wouldn t suppose so not for the average case There must be a great difference in the different children in their susceptibility to rickets

DR DARBY In that regard I wonder Dr Park if you would feel that you could express an opinion as to the number of the per cent of these children with rickets who would have had rickets if they had no become ill with chronic disease

DR PARK I can t remember the percentage of cases in which it was evident that the rickets had anteceded the illness Dr Follis can

DR FOLLIS Well we had quite a number of cases of acute illness—1 to 14 days duration—and the incidence is quite high I can t give you the exact figures The chronicity of the disease seems not to have much effect on rickets but we have a number of chronic illnesses of a month or more duration which d d not have rickets I might point out that a study like this I m afraid can t be made again because the children aren t dying the way they did before chemotherapy and I doubt if we can ever get information on the current presence of rickets Both Dr Park and I feel of course that this is the most sensitive way of determining rickets and the number of cases we found at autopsy in which rickets was diagnosed quantitatively was a small group

EXPERIMENTAL MUSCULAR DYSTROPHY

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HISTORY

SOME TWELVE YEARS AGO, conclusive evidence was obtained in Professor McCollum s laboratory that nutritional muscular dystrophy in the rabbit can be prevented or cured by alpha tocopherol (1) This *dramatic disease, characterized by rapid and massive necrosis of the* voluntary muscles, had been first produced in 1931 in rabbits and guinea pigs by Goettsch and Pappenheimer (2) They obtained the disease in animals fed *either a natural food diet* or the same diet treated with etherial ferric chloride to destroy vitamin E The untreated diet contained vitamin E as shown by its ability to cure sterility in vitamin E deficient female rats and to maintain spermatogenesis in males Moreover, the oral administration of wheat germ oil of proven potency did not prevent dystrophy in rabbits and guinea pigs fed the ferric chloride treated diet Goettsch and Pappenheimer recognized that the production of dystrophy on a diet low in E was non sequitur since dystrophy could also be produced on a diet containing E, and so they headed a main section of their paper, Elimination of Vitamin E as a Factor in the Production of Muscular Dystrophy They concluded that the lesions, must be referred to some still unknown factor I mention this because following our report the work of Goettsch and Pappenheimer began to be referred to by others as demonstrating that E deficiency was the cause of muscular dystrophy This is still the case today This is unfortunate for it dismisses the original findings of Goettsch and Pappenheimer as being due to misinterpretation or error and eliminates other possible explanations of their results

In 1936 Pappenheimer and Goettsch (3) described the transmission of nutritional muscular dystrophy to rabbits *in utero* and

wrote, It is therefore not justifiable at the present time to speak of this condition as a deficiency disease But evidence is accumulating that the addition of vegetable oils (soy bean oil, cotton seed oil) to the experimental diet exercises a protective effect In the same year, Madsen (4) obtained partial protection with cotton seed oil However, its unsaponifiable fraction was found to be inactive, a result in accord with the conclusion of Goettsch and Pappenheimer that vitamin E was not a factor in the production of the disease

Morgulis and Spencer (5) published their first study on the cause of experimental dystrophy in 1936 and expressed the opinion that *their work confirmed the conclusion of Goettsch and Pappenheimer* The former workers wrote that Neither the presence of ferric chloride itself in Diet 13 nor the absence of vitamin E from this diet can be regarded as the principal factor in the production of nutritional muscular dystrophy Evidence was presented that there were at least two factors involved and that both were essential for the prevention of muscle lesions in rabbits Subsequently, Morgulis, Wilder, and Eppstein (6) reported that the water soluble factor was present in an alcoholic extract of defatted wheat germ and that the fat soluble factor was contained in the unsaponifiable fraction of wheat germ oil and cotton seed oil They suggested that the fat soluble factor was perhaps even identical with vitamin E When both factors were fed to dystrophic rabbits, cures were obtained However, the cures were but temporary and even though therapy with both factors was continued, the disease recurred in about four weeks Moreover, Thomas and coworkers (7) reported that rabbits on a vitamin E deficient diet bore young over a two year period, a performance that appeared to exclude the possibility of E deficiency being the cause of muscular dystrophy in this species

In 1928 Evans and Burr (8) observed the occurrence of paralysis in the suckling young of female rats fed a diet low in vitamin E Although the paralysis was generally fatal, some animals exhibited spontaneous recovery and, except for sterility, remained asymptomatic on E deficient diets In 1935 Ringsted (9) and Blumberg (10) described paresis of the hind limbs of adult rats maintained on E deficient diets for many months Einarson and Ringsted (11)

ascribed the "old age" paralysis to changes in the central nervous system and attributed the muscle lesions that eventually developed to the nervous changes It was not until ten years after the initial observation of Evans and Burr, that Olcott (12) noted extensive degeneration of the voluntary muscles in the suckling rat Nerve lesions in vitamin E deficient suckling rats[1] had previously been reported by Lipshutz (13) In contrast to the findings with rats, Rogers, Pappenheimer and Goettsch (14) observed no changes in the nervous system of dystrophic rabbits and guinea pigs and concluded that the muscle lesions were primary Similar observations were made by Chor and Dolkart (15)

Our interest in neuromuscular disorders was stimulated by the observation (16) that the "old age" paralysis in vitamin E deficient rats was prevented by a highly active vitamin E preparation (17) Moreover once the paralysis had begun, the administration of the vitamin E concentrate arrested its further development and produced a gain in weight (18) These results and the availability of our highly active vitamin E preparation prompted us to investigate the possible involvement of vitamin E in the muscular dystrophy of rabbits despite the negative evidence that had accumulated since the initial work of Goettsch and Pappenheimer

NUTRITIONAL FACTORS

Vitamin E

In the early experiments, difficulty was encountered in diagnosing muscular dystrophy and in following its progress Young rabbits that were growing well, suddenly developed diarrhea, lost weight, and died within several days A method was therefore sought that would enable us to detect the onset of the disease It was found that creatinuria developed prior to loss of weight, loss of appetite and the appearance of physical symptoms By following creatine excretion food consumption and body weight, criteria were established

[1] This and the differences described above distinguished the condition in suckling and old rats from the dystrophy of Herbivora For a fuller discussion of the symptoms of vitamin E deficiency in the rat see Mackenzie C G *Vitamin E* Ann N Y Acad Sci 52 202 (1949)

which enabled us to predict with considerable certainty, the onset of the disease and its progress to the acute and fatal phase

With these criteria as a basis for diagnosis, we were able to produce cures in severely dystrophic rabbits by the administration of wheat germ oil or its unsaponifiable fraction Moreover, the vitamin E concentrate fed at a level of 5 mg per day cured rabbits suffering with severe dystrophy Rat assays carried out at Johns Hopkins and

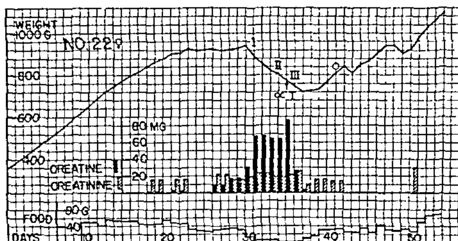


FIG 1 Cure of nutritional muscular dystrophy by alpha tocopherol The weight of the rabbit is represented by the solid line and the stage of dystrophy by the Roman numerals Daily creatine and creatinine excretion are shown by the solid and hatched columns respectively The arrow indicates the beginning of the oral administration of 5 mg of alpha tocopherol per day
From Mackenzie and McCollum *J Nut* 19 345 (1940)

by Dr Karl Mason indicated that the concentrate possessed a potency comparable to that of natural alpha tocopherol (17) The anti dystrophic activity of both the concentrate and wheat germ oil was abolished by treatment with ferric chloride No activity could be detected in the sterol or wax fractions of the unsaponifiable material

On the basis of these results, 5 mg doses of natural alpha tocopherol were administered daily to severely dystrophic rabbits Within 24 to 48 hours, there was a pronounced drop in creatine excretion, followed by a gain in weight, a resumption of food consumption, and the disappearance of physical symptoms (Fig 1) Tocopherol

therapy was continued for 7 months During this time, the animals were vigorous and grew well No muscle lesions were found at autopsy The cure was permanent and unequivocal (19) The diet employed in all of these experiments was the ferric chloride treated diet of Goettsch and Pappenheimer (Diet 13) plus a source of the water soluble factor of Morgulis and coworkers (6) The vitamin E preparations were administered orally and food was with held for several hours before and after supplementation

Water Soluble Factor

Attention was next turned to the nature of the antidystrophy water soluble factor When rabbits were allowed to develop dystrophy on Diet 13 in the absence of the water soluble factor, we found that the oral administration of alpha tocopherol alone brought about prompt and permanent cures (20) Since the possibility remained that the water soluble factor acted synergistically with vitamin E, chronic dystrophy was produced in a group of animals by feeding them subminimal doses of alpha tocopherol These animals grew well, were vigorous, and to all appearances, normal However, they exhibited a continuous creatinuria and possessed microscopic muscle lesions The incorporation in the diet of the water soluble factor, or its administration by mouth, had no effect on the creatinuria or muscle lesions The abnormal excretion of creatine was abolished by increasing the dose of alpha tocopherol It was concluded that the water soluble factor was not required for the prevention of dystrophy and that it did not act synergistically with vitamin E

When vitamin E, in the form of wheat germ oil, *was incorporated in the experimental diet* (Diet 13) instead of being administered by mouth, it failed to prevent the development of severe dystrophy However, the addition to the diet of wheat germ oil plus a source of the water soluble factor (defatted wheat germ) resulted in complete protection against creatinuria and the other symptoms of the disease Defatted wheat germ alone was without effect These results, which are summarized in Table 1, indicate that the water soluble factor is in reality an antioxidant that protects vitamin E from destruction by ferric chloride treated diets The nature of the antioxidant(s) in defatted wheat germ merits further attention

While this series of experiments offers a possible explanation for the failure of Goettsch and Pappenheimer to cure or prevent dystrophy with wheat germ oil, it should be recalled that they also fed wheat germ oil by mouth No experiments have been performed as yet that show the destruction of appreciable quantities of orally administered tocopherol in the gut by Diet 13 under such conditions

TABLE 1

RESPONSE OF DYSTROPHIC RABBITS TO VITAMIN E AND THE WATER SOLUBLE FACTOR

Supplement	Route of Administration Oral	Diet
Vitamin E	+	—
WSF *	—	—
Vitamin E + WSF	+	+
Subminimal † Vitamin E + WSF	—	—

* Water Soluble Factor

† A level of vitamin E producing good growth but insufficient to prevent creatinuria

The possibility remains that their animals were deficient in still another factor necessary for the prevention of dystrophy The fact that Goettsch and Pappenheimer produced dystrophy on a natural food diet containing vitamin E lends support to this possibility

Our demonstration of the cure of dystrophy with alpha tocopherol was confirmed by the independent work of Morris (21) with rabbits and by the experiments of Shimotori, Emerson, and Evans (22) with guinea pigs In a concomitant publication, Goettsch and Ritzmann (23) reported the prevention of dystrophy in the suckling rat by alpha tocopherol They mentioned that it would be of interest to feed alpha tocopherol to Herbivora

Cod Liver Oil

In 1932 Woodward and McCay (24) reported the production of muscular dystrophy in Herbivora fed synthetic diets Extensive investigations on the cause of the disease were carried out at the Cornell University Agricultural Experiment Station during the next 6 years and were the subject of memoirs by Madsen, McCay, and Maynard (25) and Davis, Maynard, and McCay (26) It was found

that the disease was produced in a variety of species when cod liver oil was fed to animals maintained on a purified diet or when the oil was added to a natural food diet consisting of equal parts of alfalfa and oats Dystrophy was also produced when vitamins A and D were supplied as "concentrates," but not when they were supplied in the form of carotene and irradiated yeast

Our experiments (19) and those of Shimotori, Emerson, and Evans (22) showed that alpha-tocopherol could prevent muscle lesions in Herbivora fed diets containing cod liver oil Two questions called for definitive answers, first, whether or not E deficiency per

TABLE 2

PRODUCTION OF MUSCULAR DYSTROPHY IN THE ABSENCE OF ANIMAL FAT

Young rabbits were placed on the experimental diets at approximately 400 g in weight

Diet	Appearance of Symptoms	Time of Maximum Weight	Microscopic Muscle Lesions
	Days	Days	
Fat free	30	70	++
"	21	70	++++
	17	32	++++
	29	37	++++
Lard	22	21	++
	16	26	++
	28	70	++++
	24	27	++

From Mackenzie Mackenzie and McCollum, J Nut, 21, 225 (1941)

se, in the absence of animal fat, would lead to the rapid development of severe and fatal dystrophy, and, second, whether alpha-tocopherol *could prevent dystrophy specifically produced by the feeding of cod* liver oil Young rabbits were placed on a diet consisting of defatted casein, dextrin, yeast, salt mixture, carotene, and calciferol with and without 8 per cent fresh lard (27) Dystrophy developed as rapidly *on the "fat free diet" as on the lard containing diet (Table 2)* or indeed as on Diet 13 itself In both instances the muscle lesions were completely prevented by the administration of alpha tocopherol It was thus shown that vitamin E deficiency, alone, without the

intervention of dietary fat, will produce severe muscle lesions in the rabbit. Evidently the rabbit is much more sensitive to E deprivation than the rat with respect to the development of severe muscle degeneration.

In the next series of experiments, the effect of cod liver oil on rabbits receiving a demonstrably adequate intake of vitamin E was studied. Young rabbits were fed the "low fat" diet described above and given oral supplements of 3 mg. of alpha tocopherol daily. One half of the animals were fed, in addition, 1 ml. of fresh medicinal cod liver oil daily immediately following the tocopherol supplement. The latter animals developed severe muscle lesions. The rabbits that did not receive cod liver oil were normal (27). Cummings and Mattill (28), Weber, Irwin, and Steenbock (29) and Mattill (30) had shown that rancid or autoxidizing fats destroy vitamin E in the diet. It seemed possible that in our experiments, the cod liver oil was destroying vitamin E in the gut, and that feeding the tocopherol twenty four hours apart from the cod liver oil might afford protection. A group of animals was fed the "fat free diet" plus 6 mg. of alpha tocopherol every other day. No microscopic lesions developed. A second group of rabbits was fed 2 ml. of cod liver oil together with the vitamin E. As was to be expected from our previous results, all of the animals developed severe muscle lesions. A third group of animals was fed tocopherol and cod liver oil twenty four hours apart to prevent their mixing in the gut. To our surprise, all of these rabbits developed severe muscle lesions (Table 3). To check on the rate of absorption of the cod liver oil, 2 ml. portions were saturated with Scarlet R and administered by stomach tube. Four hours later the mesenteries, beginning at the entrance of the common bile duct into the duodenum, were brightly stained. Small droplets of stained fat were seen in the duodenum and to a lesser extent in the small intestine. Practically none were present in the large intestine. Twenty four hours after its administration none of the dyed fat could be detected in any part of the gut or mesenteries. From these results, it seemed unlikely that alpha tocopherol and cod liver oil fed 24 hours apart were in contact with each other in the gastro intestinal tract. *Apparently the oil was inactivating the vitamin*

in the body itself When the level of tocopherol, administered on alternate days to the cod liver oil was raised to 40 mg , the muscles were normal

TABLE 3

PRODUCTION OF DYSTROPHY BY COD LIVER OIL FED SEPARATELY FROM VITAMIN E

Rabbits weighing 400 g were fed the purified low fat diet for 2 to 3 months Supplements were administered orally 3 times a week

Supplement α tocopherol	CLO	Day Administered	Daily Weight Gain	Microscopic Muscle Lesions
mg	ml		g	
6	0		12 1	—
6	2	same	10 8	++±
6	2	alternate	11 8	++
40	2	alternate	12 8	—

From Mackenzie Mackenzie and McCollum *Science* 94 216 (1941)

These experiments (31) provided conclusive evidence that cod liver oil can induce vitamin E deficiency in rabbits receiving an other wise adequate intake of the vitamin The muscle lesions were indistinguishable from those produced on a purified vitamin E deficient diet and they were completely prevented when the E intake was of sufficient magnitude It was known from the classic work of Mattill and his school (32) that tocopherol functions as an antioxidant for fat *in vitro*, a process in which the tocopherol itself is inactivated The experiments described above indicated that toco pherol also functions as an antioxidant *in vivo* Furthermore, they showed that the greater toxicity of cod liver oil for Herbivora as compared to the rat is due, in part at least, to the relatively mild symptoms produced in weanling and young adult rats by vitamin E deficiency

Other Dietary Factors

From the beginning of our work on muscular dystrophy, we were struck with the paucity of information concerning the morphology of the skeletal muscles in nutritional diseases in general Conse quently, in 1940 a study was begun of the skeletal muscle in a number

of deficiency diseases, produced singly and in conjunction with vitamin E deficiency The rat was chosen as the experimental animal because of the knowledge that prevailed concerning its nutritional requirements, and because the infrequent muscle lesions in young vitamin E deficient rats permitted an increase in their number to be readily detected Young rats were fed the basal diet (Table 4)

TABLE 4

Sucrose	600
Salts	60
Casein	200
Lard	40
Cystine	0 5
Choline chloride	0 25
Vitamins *	

* Thiamin 5 mg riboflavin 5 mg pyridoxine 5 mg, calcium pantothenate 10 mg, inositol 5 mg p amino benzoic acid 5 mg nicotinic acid 10 mg haliver oil fortified with viosterol 13 drops

with one of the vitamins omitted When symptoms had developed, the vitamin was administered in quantities that maintained a chronic deficiency After 16 to 20 weeks, the vitamin was withdrawn and the acutely deficient animals were sacrificed at 20 to 30 weeks In the experiment on protein deficiency, 10 per cent yeast was incorporated in the diet, the B vitamins and cystine were omitted, and the casein was reduced to 8 per cent At 9 weeks, the casein was completely withdrawn All changes were made by adjusting the sucrose content of the diet The protein deficient rats lost only a little weight during the remaining 3 months of the experiment

The results of these experiments are summarized in Table 5 [*] Although the vitamin E deficient animals exhibited no physical symptoms, their muscles showed a reduction in creatine, an increase in chloride, and widely scattered lesions (Figs 2 and 3) in confirmation of the results of Knowlton, Hines, and Brinkhous (33) None of the other deficiencies produced these changes with the exception of riboflavin deficiency in which there was an increase in

[*] Earlier publication of these results was prevented by the war

muscle chloride from a normal value of 10 m eq to 14 m eq although microscopic lesions were not discernable

TABLE 5

DIET AND MUSCLE DAMAGE

Deficiency	Microscopic Muscle Lesions	Cl	Creatine
		m eq	mg %
None	—	10	414
Vitamin E	±	14	367
Thiamin	—		
Riboflavin	—	16	
Pantothenic	—	10	
Pyridoxine	—	10	433
Vitamin A	—	11	400
Protein	—	12	450
Vitamin E + thiamin	±		
+ riboflavin	±	19	
+ pantothenic	+	14	
+ pyridoxine	+++	22	339
+ vitamin A	+++	20	312
+ protein	+++	22	316

Pyridoxide, protein, and vitamin A deficiency all intensified the changes associated with vitamin E deficiency The severity of the microscopic lesions (Figs 4 and 5) approached those observed in vitamin E deficient rabbits Krakower and Axtmayer (34) had previously found that a combined deficiency of vitamins A and E in the young adult rat produced severe muscle lesions that were pre vented by alpha tocopherol That these results were not due to general malnutrition is evidenced by the failure of thiamine, ribo flavin, or pantothenic acid deficiency to intensify the muscle changes seen in E deficiency

These experiments have interesting implications for the human, for they show that E deficiency, even in a resistant animal, may produce extensive muscle damage when combined with other specific dietary disorders Some years ago Blackfan and Wolbach (35) described Zenker s degeneration in vitamin A deficient infants In

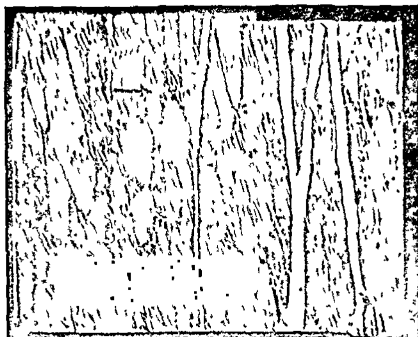


Fig 2 Typical muscle lesion in a rat maintained on a vitamin E deficient diet One such lesion is commonly found in several low power fields See text for experimental details



Fig 3 Large muscle lesion in a rat maintained on a vitamin E deficient diet Dark staining nuclei are associated with hyalinized portions of protoplasm Lesions of this size were not found in most E deficient rats See text for experimental details

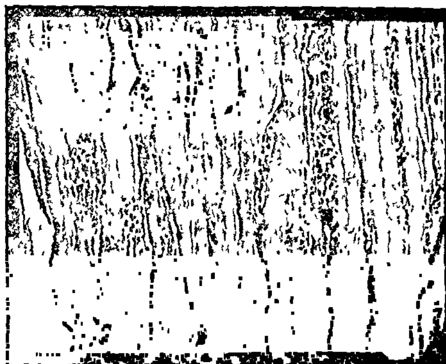


Fig 4 Muscle lesions in a rat fed a diet deficient in vitamin E and pyridoxine See text for experimental details



Fig 5 Muscle lesions in a rat fed a diet deficient in vitamin E and protein See text for experimental details

produced by at least two different deficiency diseases. Moreover, they suggest that wrist stiffness may not be a separate disorder, but rather a gross manifestation of muscle degeneration. The Cornell Experi-

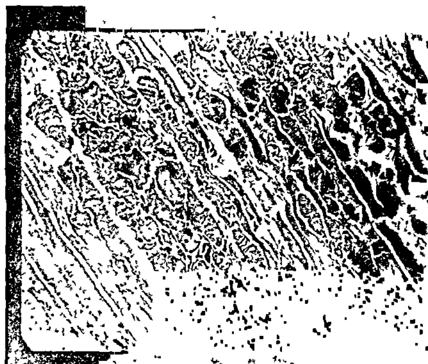


FIG 6 Muscle lesions obtained by Hanlon and coworkers (41) in a guinea pig deficient in the antistiffness factor

TABLE 6

EFFECT OF ERGOSTANYL ACETATE ON MUSCLE LESIONS IN GUINEA PIGS

Supplement	Muscles Examined	Incidence of Lesions ++ to +++	0 to +
		%	%
None	36	53	47
Ergostanyl acetate	22	4	96

From Hanlon, Foot, Travell, and Rinzler, *Federation Proc* 9, 282 (1950)

ment Station workers (25, 26) repeatedly referred to stiffness in their rabbits and guinea pigs fed cod liver oil In our original publication on the cure of dystrophy in rabbits (19), we described stiffness of the front legs as the first physical symptom to be observed.

This stage of dystrophy, stage II, gave way in several days to stage III dystrophy in which the animals became very weak and suffered a marked loss of body tonus The transitory nature of the stiffness may be due to the rapid development of muscle degeneration in E deficient rabbits as compared with its slower development in ergostanol deficient guinea pigs

It is curious that Van Wagtendonk and coworkers (37) found a decline in the creatine phosphate of muscle, but no creatinuria in their antistiffness deficient guinea pigs This seemingly contradictory finding requires clarification Experiments are needed in which both the tocopherol and ergostanol content of the same diet can be varied at will The present work still leaves something to be desired in eliminating E deficiency as the cause of muscle damage in the antistiffness syndrome In the face of the history of nutritional muscular dystrophy, this should not be done lightly Meanwhile, one wonders if the failure of Goettsch and Pappenheimer to cure dystrophy in their animals can have been due to the existence of a concomitant deficiency in ergostanol

Pathology and Symptomatology

The most striking microscopic change in the muscles of vitamin E deficient Herbivora is coagulative or hyaline necrosis of the protoplasm resembling the lesions described in the Dresden epidemic of typhoid fever by Zenker (42) in 1863 The coagulation may involve masses of fibers or portions of a muscle fiber, the remainder of the cell retaining its normal striated appearance Accumulations of dark staining nuclei appear in clefts in the hyaline masses (Fig 7) Pappenheimer is of the opinion that nuclear multiplication follows necrosis and is associated with regeneration of the muscle (2, 43), while Chor and Dolkart believe that nuclear proliferation is the first discernable degenerative change (15) Be this as it may, the degeneration spreads with explosive rapidity in severe E deficiency Phagocytes appear and the necrotic material is removed As the disease progresses great masses of cellular elements dominate the picture Calcification of the necrotic tissue is a variable and secondary phenomena

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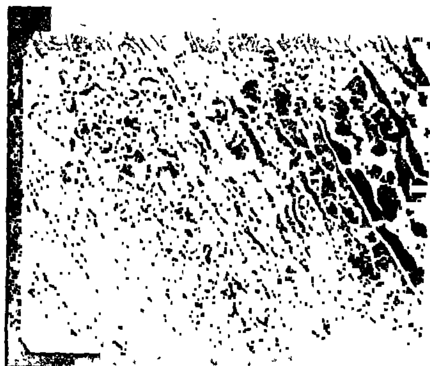


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Quite different pathological pictures can be obtained by varying the intensity and duration of vitamin E deficiency Rabbits maintained on a suboptimal intake of tocopherol one which permits good growth and vigor but does not prevent creatinuria show upon biopsy a picture dominated by healthy muscle fibers sprinkled with fibers containing hyalinized areas imbedded with nuclei Massive necrosis



Fig 7 Muscle lesions in vitamin E deficient rabbit showing accumulations of darkly staining nuclei and hyalinization and fragmentation of protoplasm Sharp cross striations are present in some of the necrotic tissue and in adjacent fibers

and large accumulations of cellular elements are lacking Repair is evident and it appears that the processes of degeneration and regeneration have reached an equilibrium (20) If on the other hand rabbits are maintained in a state of severe E deficiency the pathological picture comes to resemble progressive muscular dystrophy in man The muscle mass is largely replaced by adipose and connective tissue containing a few atrophied muscle fibers These fibers are not hyalinized or necrotic and retain their cross striations

Prolonged severe E deficiency may be produced by administering

tocopherol parenterally to dystrophic rabbits (41) There is no fall in creatine excretion or remission of physical symptoms (Fig 8) However, in contrast to untreated animals, the rabbits continue to eat, gain weight slowly, and life is greatly prolonged The process of muscle degeneration, however, is not arrested We have seen rabbits practically prostrate, with most of their skeletal muscle mass lost, continue to eat and maintain their weight for several months

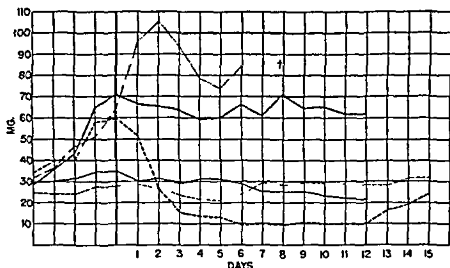


FIG 8 Creatine excretion in mg per day of dystrophic rabbits given 20 mg of alpha tocopherol in peanut oil orally or subcutaneously at 0 days The creatine excretion of untreated animals is shown in the uppermost line † indicates the time of death The heavy and thin solid lines represent the creatine and creatinine excretion respectively of injected animals The creatine excretion was maintained at the pre treatment level and the creatinine excretion slowly declined The heavy and thin broken lines represent the creatine and creatinine excretion of animals receiving a single oral dose of alpha tocopherol

From Mackenzie and McCollum *Proc Soc Exp Biol Med* 48 642 (1941)

At autopsy, it was found that their leg muscles had been replaced almost entirely by fat and connective tissue (Fig 9) One may ask whether muscle degeneration kills untreated rabbits or whether death is due to some other change that is prevented by exceedingly small amounts of vitamin E Injected tocopheryl phosphate is effective in curing muscular dystrophy in rabbits (44) These results speak against any conclusions being drawn from the parenteral administra

tion of tocopherol to man, a fact that has been frequently overlooked in clinical experiments.

One of the most dramatic aspects of experimental dystrophy is the remarkable regeneration of muscle following E therapy. Rabbits have been cured of as many as 5 successive attacks of dystrophy (Fig. 10) in each of which they verged on complete collapse (Stage III) and exhibited an intense creatinuria (45). Two to 4 weeks



FIG 9 Thigh muscle from a rabbit with prolonged dystrophy showing the increase in connective tissue The remaining muscle fibers have retained their cross striations and show no hyalinization

after the final attack, the muscles of these animals were essentially normal, microscopic examination revealed no necrosis, accumulation of cellular elements, increase in fat or striking increase in connective tissue The regenerative power of the muscle in the presence of a positive stimulus appears to be almost unlimited

Inasmuch as testicular degeneration is the most prominent sign of E deficiency in the young male rat, it is of interest that testicular damage was not encountered in our rabbits (46), even when they

experiments with rabbits that creatinuria preceded the appearance of physical symptoms or weight loss (19) Recently Melville and Hummel (56) have made the important observation that creatinuria and loss of muscle creatine in the rabbit precede the appearance of

TABLE 7

CHANGE IN MUSCLE COMPOSITION IN DYSTROPHIC RABBITS

Increased	Decreased
Na	K
Cl	Mg
Ca *	P, acid soluble
P total *	Creatine P
Cholesterol	Creatine
Ribonucleic acid	Glycogen
Desoxyribonucleic acid	Total N
	Glutamine †

* Increased only in calcified muscle
† Decrease slight in rabbits, greater in guinea pigs

microscopic lesions The concentration of guanidoacetic acid in the muscle was unchanged, there was a moderate increase in the quantity excreted in the urine A decrease in glutamine in the muscle of dystrophic guinea pigs has been reported by Roderick (57) The blood of dystrophic rabbits was studied by Morgulis and Spencer (58) who found no striking changes except a rise in cholesterol There was perhaps a small rise in lipid phosphorus Melville and Hummel (56) report an increase in blood creatine in incipient dystrophy

CHEMISTRY OF URINE

In a wasting disease, the greatest increase in excretory products will be found in those compounds that cannot be metabolized by the animal Hence, one might expect an increase in the excretion of K and P, and this is the case (59) On the organic side, the release of creatine from degenerating muscle should result in creatinuria since creatine is not metabolized (60, 61), at least not by the rat, and this is the case Inasmuch as creatinine is derived from creatine

(62), the excretion of creatinine would be expected to fall when the dystrophy is of appreciable duration This is so (14) although it has been overlooked when attention is restricted to our earlier, short term experiments In prolonged dystrophy, a definite fall in creatine excretion also occurs (44) in conjunction with a great loss of muscle tissue and its replacement by adipose and connective tissue

With respect to the other organic constituents of muscle, they will appear in the urine when their rate of release from degenerating muscle exceeds their rate of metabolism Minot and Grimes (63) have reported the presence of a pentose phosphorus complex in the urine of dystrophic rabbits The pentose has been isolated and identified as ribose, which may have been derived from ribose nucleic acid, ATP, or some other nucleotide The appearance of this complex in the urine is preceded by the creatinuria of dystrophy In this connection, Young and Dinning (55) found an increase in allantoin excretion in dystrophic rabbits At the conference on vitamin E held at the New York Academy of Sciences, Ames (64) stated in the discussion that in rabbits deprived of vitamin E, the excretion of amino nitrogen and ascorbic acid was elevated within one week, thus preceding the advent of creatinuria

While the observed chemical changes in muscle, blood and urine are of diagnostic value and of interest in obtaining a picture of a degenerative process, it can hardly be hoped that a chemical study of *pathological tissue* will disclose the underlying chemical lesion that initiates the subsequent changes in cell chemistry and morphology That is like hoping to find a needle in a haystack Never theless, the early release of creatine from muscle in vitamin E deficiency focuses attention on the lack of information concerning the location or the binding of the creatine molecule in the cell Possibly vitamin E plays a role in the latter process and perhaps the binding of creatine phosphate is essential for maintaining the structural integrity of muscle protein Recent work suggests a disturbance in nucleic acid or purine metabolism However, it should be recalled that an increase in nuclei, monocytes, polymorphs, etc is one of the early microscopic changes observed in dystrophic muscle Moreover, muscle regeneration is occurring side by side with degeneration All

of these changes would tend to increase the concentration of nucleic acids Obviously, a thorough study of muscle in E deficiency *prior to the appearance of lesions* is needed Results obtained on dystrophic muscle may always be secondary to tissue degeneration They confuse the literature and cannot of themselves be interpreted

OXYGEN CONSUMPTION

In 1934 Victor (65), working in Pappenheimer's laboratory, found an increase in the oxygen uptake of muscle from dystrophic rabbits Similar results were described by Madsen (4) two years later In 1940 Friedman and Mattill (66) reported that muscle *strips* removed from rats maintained on an E deficient diet for 6 months exhibited a high oxygen consumption It was found that alpha tocopherol acetate administered orally lowered the oxygen uptake Since infre quent muscle lesions are found in 6 month old rats, these results suggested that an increased oxygen consumption preceded necrosis and cellular infiltration

TABLE 8

OXYGEN UPTAKE OF MUSCLE STRIPS FROM YOUNG VITAMIN E DEFICIENT RATS *

Diet	Supplement	Q_{O_2}
E deficient	None	6 3
	α tocopherol	4 4
Stock	None	6 8
	α tocopherol	6 4

* From Kaunitz and Pappenheimer *Amer J Phys* 138 328 (1943)

Kaunitz and Pappenheimer (67) made a thorough study of the oxygen uptake of muscle *strips* from E deficient nursing rats and found an increase in the consumption of muscles from the deficient rats compared with the muscles from rats given a single dose of alpha tocopherol (Table 8) The increased oxygen con sumption occurred in the absence of microscopic lesion and the respiration of the liver was unaltered Of general interest, is the finding that the respiration of muscles from rats fed a stock diet

equaled that of the *vitamin E deficient animals* Since vitamin E did not lower the muscle oxygen consumption of the stock rats, it appears that vitamin E is not the only dietary factor concerned with muscle respiration

At the same time, Houchin and Mattill (68) reported an increased oxygen consumption in muscle *slices* from E deficient rats, rabbits, and hamsters The oral administration of tocopherol or its phosphate brought about a fall in oxygen consumption within 10 hours Intra venous tocopheryl phosphate reduced the oxygen consumption within one hour as shown by biopsy experiments Surprisingly the already reduced creatine content of the muscle fell to a still lower level In the same series of papers, Houchin (69) described a depression of oxygen consumption when tocopherol phosphate was added to muscle slice *in vitro* The significance of these results is difficult to evaluate inasmuch as Hummel and Basinski (70) in Mattill s labora tory have been unable to demonstrate a reduction in the oxygen uptake of muscle *slices* from dystrophic rabbits Hummel and Basinski report that muscle *strips* from the semitendinosus of dystrophic rabbits possessed a decidely high oxygen uptake Contrary to the results of Houchin (69), in none of their preparations, normal or dystrophic, slice or strip, did the *in vitro* addition of alpha tocopheryl phosphate depress the oxygen consumption Recently, Hummel and Melville (71) have confirmed the increased oxygen uptake of muscle *strips* (psoas muscle) from E deficient rabbits and found that this change precedes the appearance of microscopic lesions There was no change in the R Q or rate of glycolysis at this time

While these experiments leave no doubt that the oxygen consumption of muscle is increased in E deficiency, they also emphasize the need for improved techniques and methods in the study of muscle respiration and metabolism In the experiments of Kaunitz and Pappenheimer (67), 38 measurements were made on muscle strips from stock rats The mean Qo was 6 8, the extremes, 3 9 to 12 2 and the standard deviation 1 83 Particular attention should be paid to the immediate nutritional history of the animal his activity, the method of killing the preparation of the muscle and so on The difficulty in obtaining comparable results with muscle from different

animals has been one of the major obstacles in elucidating the anti dystrophy action of tocopherol

Inasmuch as increased oxygen uptake precedes the development of lesions in the rat and rabbit, it is important that such measurements, together with chemical analysis, be extended to species which do not show Zenker's degeneration The work of Kaunitz and Pappenheimer (67) suggests that in the E deficient chick there may be an increase in the oxygen consumption of muscle This observation should be confirmed and extended On the other hand, Victor (65) reported that in the E deficient duckling with severe muscle degeneration, there was no increase in oxygen consumption Muscle creatine was greatly decreased This experiment must be repeated if we are to have a true understanding of the significance of increased oxygen consumption in muscular dystrophy

A large increase in respiration is a striking response to the absence of a vitamin or any other dietary factor In the case of E deficiency, it is compatible with the antioxidant activity of tocopherol Some years ago, Hastings, Muus, and Bessey (72) reported an increased oxygen uptake in the diaphragms removed from riboflavin deficient rats It is interesting that in our studies, described earlier, only E and riboflavin deficiencies produced an increase in muscle chloride

The effect of E deficiency on the oxygen consumption of the whole rat has been investigated by Kaunitz and Pappenheimer (67) They found an increase in total oxygen consumption commensurate with the increased rate of respiration of the skeletal muscles Zieler, Folk, Eyzaguirre, Jarcho, Grob, and Lilienthal (73), produced a 12 per cent reduction in the basal oxygen consumption of normal rats by the oral administration of alpha tocopheryl phosphate The parenteral administration of this compound depressed the oxygen consumption of the diaphragm by 25 per cent, but had no effect on the respiration of liver or brain slices Drowsiness, ataxia, and flaccidity followed the injection The authors believe that the active agent is free tocopherol, formed from the hydrolysis of the phosphate ester in the body, and have obtained similar results by feeding large doses of alpha tocopherol acetate (74)

ENZYMES

The enzyme experiments may be divided into 2 parts, studies on the activity of enzyme systems prepared from E deficient animals, and studies on the effect of the *in vitro* addition to enzyme preparations of alpha tocopherol and its derivatives Much of the latter work has involved the use of alpha tocopheryl phosphate The currently prevailing view is that this compound acts on enzyme systems as a detergent in a nonspecific manner Accordingly, many of the experiments with the phosphate will not be discussed here and the reader is referred to the review of Ames and Harris (75) for additional references on the subject It should be pointed out that the possession of apparently nonspecific activity by a class of compounds such as detergents does not exclude the possibility that only one of them exists in living cells and hence exhibits this activity *in vivo* as a specific function While this may be the case with tocopheryl phosphate evidence is lacking at present that it occurs in the animal body

The lead in studying enzyme systems from dystrophic muscle was taken in Mattill s laboratory and it has continued to provide most of our knowledge of the subject In 1942 Houchin (69) reported a 160 per cent increase in the succinic oxidase system in the muscles of dystrophic hamsters and rats This abnormal activity was inhibited by alpha tocopheryl phosphate but not by alpha tocopherol itself Soon after Govier Bergmann and Beyer (76) found that alpha tocopheryl phosphate inhibited the succinic oxidase system prepared from the muscles of normal rats However Basinski and Hummel (77) found no increase in the muscle succinic oxidase system in dystrophic hamsters Tocopheryl phosphate inhibited to an equal degree the activity of the system prepared from either normal or dystrophic muscle

Ames (78) studied this problem and concluded that the alpha tocopheryl phosphate inhibition of the succinic oxidase system was due to the removal of calcium ions The inhibition could be relieved by the addition of calcium chloride He proposed the following mechanism the removal of calcium ions reduces the activity of

diphosphopyridinenucleotidase and the resulting accumulation of DPN increases the concentration of oxalacetate which in turn inhibits succinic dehydrogenase

Recently Rabinovitz and Boyer (79) studied the effect of alpha tocopheryl phosphate on the rat heart succinic oxidase system They present evidence that tocopheryl phosphate acts primarily on the component that couples the oxidation of succinate with the reduction of cytochrome c, and that it possesses a lesser inhibitory effect on succinic dehydrogenase and cytochrome oxidase themselves Similar effects were obtained with sodium dodecyl sulfate and these workers concluded that the action of tocopheryl phosphate is due to its detergent properties and is not necessarily related to its action as a vitamin The same conclusion has been reached by Ames and Rinsley (80) and by Zierler *et al* (74)

Hummel, in Mattill s laboratory (81) has studied the metabolism of glycerophosphate and fructose 1 6 diphosphate in homogenates prepared from guinea pig and hamster muscle The formation of lactic acid from these substances was decreased in E deficiency under both aerobic and anaerobic conditions Vitamin E deficiency de pressed phosphocreatine formation in all of the experiments Hummel concluded that these alterations were indirect effects of vitamin E deficiency

Zierler, Folk Eyzaguirre, Jarcho, Grob, and Lilienthal (73) approached the problem in a different fashion They injected normal rats with alpha tocopheryl phosphate and presented some evidence that this treatment impaired glycogen phosphorolysis in the dia phragm In subsequent experiments (74), the diaphragms were permitted to use up endogenous substrates by preliminary incubation for one hour and specific substrates were then added The dia phragms from the injected rats removed less glucose 1 phosphate and produced less lactic acid than did similar preparations from untreated animals When fructose 1 6 diphosphate was used as a substrate, there was no difference between the injected and the control animals in the rate of disappearance of the substrate, in the production of lactic acid, or in oxygen consumption According to these results, the injection of alpha tocopheryl phosphate blocks

glycolysis at the phosphoglucomutase, isomorase, or phosphohexokinase level The oral administration of large doses of alpha tocopherol acetate gave similar results It is important to determine whether or not the activity of any of these enzymes in the diaphragm is *increased* in vitamin E deficiency

In a recent communication, Barber, Basinski, and Mattill (82) have reported a decrease in the aspartic glutamic transaminase activity of skeletal muscle homogenates from guinea pigs and rabbits The activity was not restored by the addition to the preparation of pyridoxal phosphate It is tempting to speculate on the relationship between this and the observation that pyridoxine and protein deficiencies intensify the microscopic and chemical changes in the muscles of vitamin E deficient rats Perhaps the rabbit and guinea pig require vitamin E for the maintenance of minimal concentrations of pyridoxal phosphate, whereas the rat only develops a deficiency in the coenzyme in the face of a dietary deficiency of pyradoxine According to Cammarata and Cohen (83) all of the amino acids are involved in transamination reactions Consequently, a decrease in transaminase activity might lead to a decrease in amino acid concentration and protein synthesis in dystrophy and an increase in ketoacid concentration, an increase in the activity of the Krebs cycle and hence an increase in oxygen consumption

Despite the interesting results obtained by a study of enzyme preparation from dystrophic muscle, no direct evidence exists as yet that vitamin E is essential to the elaboration or maintenance of a specific enzyme or coenzyme, or that alpha tocopherol functions as a coenzyme

Activity of Tocopherol Derivatives

Many investigators have reported that the quinone and hydroquinone of alpha tocopherol are ineffective in curing sterility in the female rat and muscular dystrophy in the rabbit Recently J B Mackenzie, Rosenkrantz, Ulick, and Milhorat (84) reexamined the antidystrophy activity of these compounds The author had shown earlier (85) that antidystrophy activity can be measured by determining the duration of a fall in creatine excretion following the

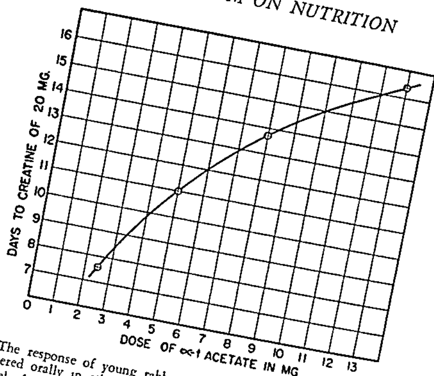


FIG 11 The response of young rabbits to D, L, alpha tocopherol The tocopherol was administered orally in ethyl laurate on the day after the creatine excretion had reached a level of 20 mg, or more The duration of the response was the number of days, following supplementation, required for the creatine excretion to again attain a level of 20 mg

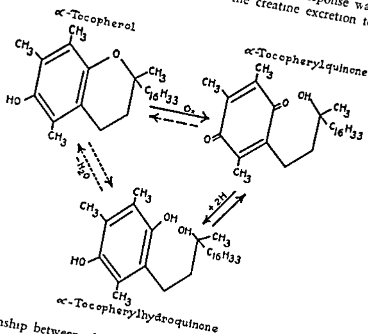


FIG 12 Relationship between alpha tocopherol and its quinone and hydroquinone

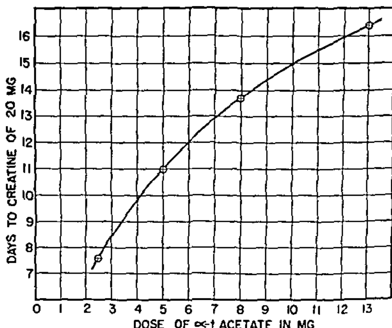


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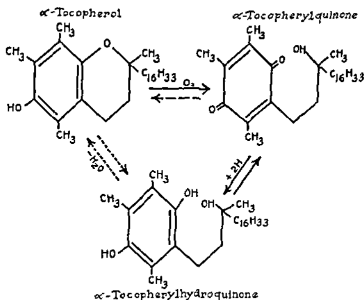


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administration of vitamin E (Fig 11) J B Mackenzie and coworkers, employing this method, found that both the hydroquinone and the quinone of alpha tocopherol (Fig 12) possess biological activity when administered in sufficiently large doses, either orally or intravenously The activity, as measured by the duration of the fall in creatinuria, was greater by the parenteral than by the oral route

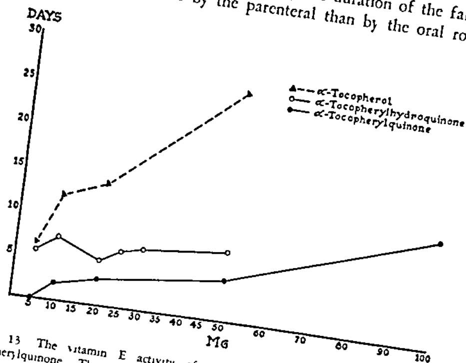


Fig 13 The vitamin E activity of alpha tocopherylhydroquinone and alpha tocopherylquinone The vertical axis indicates the number of days of suppression of an abnormal creatinuria following a single dose (horizontal axis) of the test compound injected intravenously

From J B Mackenzie *Biological Antioxidants* Transactions of the Fourth Conference Josiah Macy Jr Foundation New York (1950)

Tocopherylhydroquinone when administered intravenously in small amounts (Fig 13), possessed a potency comparable to that of alpha tocopherol In contrast to the graded response obtained with alpha tocopherol increasing the dose of the hydroquinone beyond the 5 mg level did not increase the response When injected daily at a 12 mg level the hydroquinone completely abolished the creatinuria and the physical symptoms of severely dystrophic rabbits The animals grew

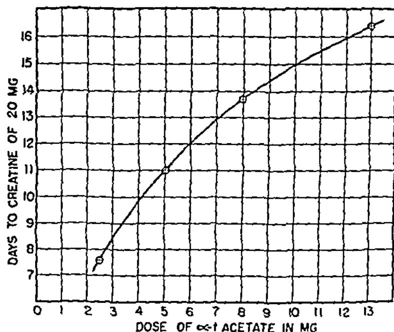


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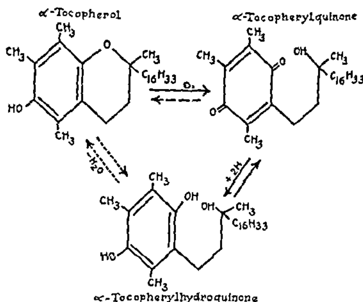


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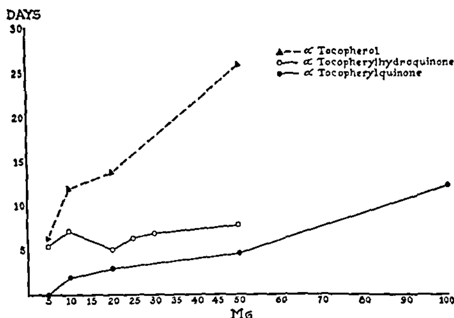


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well and were free of muscle lesions[4] The demonstration of the antidystrophic activity of alpha tocopherylhydroquinone raises many interesting questions Although its activity may be due to its conversion to alpha tocopherol in the body, *the possibility cannot be dismissed that it is active per se and that the antidystrophy activity of alpha tocopherol may be due to its conversion in vivo to the hydroquinone*

An attempt to answer this question has been made by J B Mackenzie (86) who studied the tocopherol level in the plasma of rabbits following the injection of alpha tocopherylhydroquinone The problem was complicated by the finding that rabbits were free from creatinuria and other signs of dystrophy even when the plasma tocopherol had fallen below a detectable level Thus the plasma of young rabbits fed a commercial pellet diet contained no measurable tocopherol (less than 0 05 mg per 100 ml) after the second week These animals were free of physical symptoms and creatinuria and upon autopsy, at 5 to 6 weeks, showed only an occasional damaged muscle fiber Similarly, tocopherol disappeared from the plasma of rabbits fed Diet 13 well before the appearance of creatinuria When rabbits with zero plasma tocopherol were injected with 5 mg of alpha tocopherol, the level rose to 0 4 mg per cent by the following day On the other han[illegible] ng substances were found in the tocopherol fraction of [illegible] day follo[illegible] the injection of 5 to 100 mg of alph[illegible] hydro[illegible] Since these two compounds possess [illegible] at the level these [illegible] dicate that very littl[illegible] converted to [illegible] *in vivo*

Issid[illegible] (87) have re[illegible] of doses of to[illegible] in the again found [illegible] Two poss[illegible] of biological [illegible] co[illegible] hydroquinone [illegible] le dose nancy J B M[illegible] d

[4] Dr Karl Mason [illegible] has [illegible] dystrophic effect of [illegible] adult hamsters

not stored in the rabbit and that the effect of the maximal single dose was limited to 5 to 6 days Alternatively, the hydroquinone may be totally inactive in preventing sterility in the rat despite its antidystrophy activity If this is so, it means that the antidystrophy activity of alpha tocopherylhydroquinone is not due to its conversion to tocopherol [8] Some years ago, Goettsch and Ritzmann (23) reported that the oil prepared from ferric chloride treated wheat germ prevented dystrophy in the suckling rat but did not possess antisterility activity Although the question of tocopherol requirements for these two functions was not eliminated, their experiments must be reconsidered in the light of the newer knowledge of the antidystrophy activity of the hydroquinone and quinone

A new derivative of alpha tocopherol containing one additional atom of oxygen has been prepared by Boyer (88) He has presented evidence that the compound is either 8, 9 or 9, 10 epoxy alpha tocopherol The compound is readily reduced to alpha tocopherol and it possesses biological activity of a reduced character in the rat sterility test (89) The possibility that a semiquinone radical can be formed from alpha tocopherol was investigated by Michaelis and Wollman (90) The vitamin was dissolved in a mixture of organic solvents and cooled to the temperature of liquid air When irradiated with ultra violet light, the mixture developed a color with characteristic bands The color persisted after the irradiation was stopped, but faded out when the solution melted evidence that the free radical of tocopherol had been formed

The experiments described above represent fundamental advances in the relation between the structure of tocopherol and its biological

[8] Recently we have shown in critical tests (Mackenzie J B and Mackenzie C. G, unpublished experiments) that the *daily intravenous* injection of large doses of alpha tocopherylhydroquinone does not prevent sterility in vitamin E deficient female rats These results indicate that the hydroquinone is not converted to alpha tocopherol in the animal body to a significant extent and that alpha tocopherylhydroquinone is not an antidystrophic factor by virtue of its conversion to tocopherol It appears therefore that tocopherylhydroquinone is an antidystrophy factor or vitamin itself Alpha tocopherol on the other hand is the antisterility vitamin It may also possess antidystrophic activity or alternatively it may only be the provitamin for tocopherylhydroquinone In the latter event it is possible that some human muscular dystrophies are caused by an inability to convert tocopherol to tocopherylhydroquinone or by an excessive rate of destruction of the latter compound

activity Their extension offers one of the most promising means for obtaining a picture of the action of vitamin E in living systems

Summary

The most ubiquitous response to vitamin E deficiency is the development of muscle lesions The pathology is similar to the coagulative necrosis of striated muscle described by Louis and later by Zenker in fatal cases of typhoid fever in Germany in the last century It is the same as the muscle degeneration encountered in the great influenza epidemic during World War One by MacCallum, Wolbach and Goodpasture in persons dying of pneumonia following influenza or measles and studied later in detail by Forbus (91) In these instances, the lesions had a unique distribution, being confined to the rectus abdominus The wide spread muscle lesions seen in dermatomyositis (92) appear to be identical with those produced by experimental vitamin E deficiency In both instances, they may develop rapidly and clear up with almost equal rapidity, spontaneously on the one hand and following E therapy on the other The same type of muscle degeneration has been found by Dalldorf (93) in young mice and hamsters infected with Coxsackie virus It appears that a deficiency of the antistiffness factor causes similar lesions in guinea pigs We are ignorant of the reason for the same alteration in cellular structure in these diversified diseases Perhaps, as pointed out by Dr Pappenheimer, the pathological response of muscle is limited Perhaps each of these conditions interrupts, at different points, a series of biochemical reactions necessary for the integrity of skeletal muscles Whether or not vitamin E plays a role in all of them we do not know

However, vitamin E deficiency offers a means by which this type of muscle damage can be studied under controlled conditions In Herbivora, both the extent and the appearance of the pathology may be altered by varying the degree and the duration of the deficiency Lesions may be produced in animals without the appearance of physical symptoms and without arresting growth Their presence is disclosed by the existence of creatinuria and verified by muscle biopsy

By analogy, unsuspected muscle lesions may occur in man due to avitaminosis E or other causes in the absence of gross manifestations Blackfan and Wolbach (35) described muscle lesions in vitamin A deficient infants that in retrospect seem to have been due to a concomitant vitamin E deficiency

Extensive studies on the chemistry of dystrophic muscle have yielded the findings to be expected in a degenerative disease, but they have not thrown light on the underlying chemical lesion that initiates the subsequent morphological and chemical alterations The most provocative change observed and one that precedes the appearance of microscopic lesions, is an increase in the oxygen consumption of the muscles Studies on the enzyme systems of vitamin E deficient animals have not as yet revealed the reason for the increased respiration although they have provided clues for further work A loss of muscle creatine and creatinuria also precede the onset of lesions Whether this is a fundamental disturbance or merely the reflection of a more basic one is not known More work is needed on the chemistry of the muscle in the predystrophic phase of the disease

The rabbit and Herbivora in general are more sensitive to vitamin E deficiency with respect to muscle damage than the young rat or the chick Uncomplicated vitamin E deficiency leads to extensive myodegeneration in the rabbit within a few weeks Rats fed similar diets for months show only occasional muscle lesions Consequently, the administration of cod liver oil which destroys vitamin E in the diet or in the gastrointestinal track has far more serious consequences for the rabbit than for the rat Moreover, when fed to rabbits, cod liver oil destroys vitamin E in the body

A relatively high level of cod liver oil in the rat s diet does not lead to severe muscle lesions Nonetheless severe muscle lesions may be produced in young adult rats by combining pyridoxine vitamin A or protein deficiency with vitamin E deficiency This observation together with the effect of the Coxsackie virus indicates that the young rodent is not inherently resistent to Zenker s degeneration

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A most important advance in the field of muscular dystrophy and vitamin E has been the discovery that alpha tocopherylhydroquinone is a potent antidystrophic factor but not an antisterility factor This finding raises a host of new questions Is alpha tocopherol only a provitamin (with respect to muscular dystrophy) that is converted in the body to the active hydroquinone? Is the mechanism for this conversion lacking in some human muscular dystrophies? These and other hopeful questions will be answered by further experiments and clinical tests Irrespective of their outcome, I am convinced that the body of knowledge that has been amassed by the many who have labored in this field will someday come to full fruition in its application to human disease and therapy

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A most important advance in the field of muscular dystrophy and vitamin E has been the discovery that alpha tocopherylhydroquinone is a potent antidystrophic factor but not an antisterility factor This finding raises a host of new questions Is alpha tocopherol only a provitamin (with respect to muscular dystrophy) that is converted in the body to the active hydroquinone? Is the mechanism for this conversion lacking in some human muscular dystrophies? These and other hopeful questions will be answered by further experiments and clinical tests Irrespective of their outcome, I am convinced that the body of knowledge that has been amassed by the many who have labored in this field will someday come to full fruition in its application to human disease and therapy

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E by cod liver oil, the protection of vitamin A stores by vitamin E, etc, indicate that vitamin E performs a similar function in the living organism The inhibition by alpha tocopherol of the *in vivo* formation of peroxides and free radicals from unsaturated fatty acids would lead to the destruction of tocopherol The formation of peroxides and radicals would then be accelerated and these substances might react with muscle protein to produce coagulation and necrosis If this be the case, there must be some inherent difference in metabolism between the rabbit and the weanling rat which accounts for their different reaction to uncomplicated vitamin E deficiency Perhaps the rabbit forms more peroxides from *endogenous* (and exogenous) fat than the rat, or alternatively, the young rat may begin to synthesize antioxidants that are unavailable to the rabbit and suckling rat It has recently been shown by Rumery in Mason s laboratory (94) that the resistance of the immature rat to muscular dystrophy may be overcome by raising the cod liver oil content of the diet from 6 per cent to 10 or 20 per cent Tobin (95) in Mason s laboratory has produced severe lesions in immature mice by feeding them 20 per cent of cod liver oil These results are compatible with the hypothesis that peroxides and free radicals formed from unsaturated fats are responsible for the lesions, and that tocopherol functions as a biological antioxidant to prevent their production This might be termed the unitarian theory of the mode of action of vitamin E

A number of experiments have been carried out on the theory that tocopherol may function as a coenzyme in an oxidation reduction system The results have indicated that vitamin E deficiency leads to a disturbance in carbohydrate and amino acid metabolism Whether these effects are primary or secondary to the process of muscle degeneration is not known However, the recent observation that the quinone and hydroquinone of vitamin E cure dystrophy removes a disturbing objection to the coenzyme oxidation reduction theory It is equally compatible with the view that tocopherol acts as an anti oxidant We have then as a second theory, that of the dual function of alpha tocopherol, as a biological antioxidant and as a coenzyme At the moment, there is no direct evidence that it acts as a coenzyme

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DISCUSSION

DR AMES I should like to comment briefly on our unpublished investigations referred to by Dr Mackenzie Following our report on the occurrence of aminoaciduria in progressive muscular dystrophy, attention was directed to the vitamin E deficient rabbit It was found that the removal of vitamin E from an otherwise adequate diet resulted in the appearance of a marked aminoaciduria and ascorbic aciduria This abnormal excretion preceded the characteristic creatinuria by a week or more Administration of vitamin E was followed by an immediate return to normal excretory levels of these three urinary constituents

Another experiment relating to the physiological function of vitamin E involved the preparation in vitro of a D α tocopherol protein complex About one mg per ml of D α tocopherol complexed with bovine plasma albumin was added to a functioning succinic oxidase system The initial rate of oxidation was maintained for a period of 3 hours in contrast to the normal decrease in rate of the enzyme system without added vitamin E In stabilizing the rate of this enzyme system, D α tocopherol was interpreted as functioning *in its postulated role as a physiological antitoxidant*

DR MASON I want to make one comment on species differences which Dr Mackenzie has emphasized Considering first the skeletal musculature in animals on much the same type of vitamin E deficient diet, one might list the species in order of increasing difficulty in producing dystrophic lesions much as follows—the rabbit and guinea pig, the Florida cotton rat and hamster, the rat, mouse and monkey If one considers smooth muscle, which responds to the vitamin E deficient state by pigmentation and perhaps some degeneration, such changes cannot be produced as far as we can tell in the Florida cotton rat even though it dies of dystrophy, in the hamster they involve only the urinary bladder and vascular smooth muscle, in the rat only the uterine smooth muscle and to a slight extent in the vascular smooth muscle, in the dog chiefly the intestinal musculature and in the monkey chiefly the vascular smooth muscle If one goes to the cardiac muscle, one finds a sequence of susceptibility somewhat like that for skeletal muscle, except perhaps for cattle where cardiac muscle is involved especially early

I merely wish to emphasize the striking species differences with respect to the response of three muscle types to vitamin E depletion which may in turn reflect metabolic differences usually not recognized by the biochemist or the physiologist These variations may be of some value in elucidating the functions of vitamin E

DR PAPPENHEIMER One point that I ve always found a bit mysterious and was hoping that Dr Mackenzie could throw some light upon he has referred to the massive muscle necrosis that develops in weanling rats born of E deficient mothers toward the end of the lactation period Now many of these rats don t die and, if they survive after lactation, even though the diet be rigidly deficient in E recover with extraordinary rapidity Three or four days after weaning the muscle practically resumes its normal appearance and the whole regenerative process takes place inside of a week Why? What happens in this transition between the lactating period and subsequent interval It is not many more months later that rats develop the muscular degeneration which Dr Mackenzie has described During the post lactational period they are apparently normal with complete restitution of all this degenerating muscle What could be the explanation for that?

DR MACKENZIE The situation you refer to could be explained in one of two ways—both involving a change in metabolism Either the rat, when it gets through the suckling period changes its metabolism so that it no longer produces some damaging substances at a fast rate, or, alternately, at this period the rat begins to develop other antioxidants that can function in a similar fashion to vitamin E These could be in the nature of sulfhydryl compounds, for example

DR PAPPENHEIMER I should like to ask if there is any evidence of the synthesis of tocopherol by intestinal flora There might be a change in the intestinal flora at that stage

DR MACKENZIE I tried to examine that possibility some time ago Our experiments resulted in the discovery of the anti thyroid action of the sulfonamides and thioureas and we never got back to the original question I think that there s no clear evidence so far that tocopherol is synthesized by any bacteria It s still a possibility but I rather think that some other compound begins to be elaborated by the rat in a greater quantity and takes over a function that can also be fulfilled by tocopherol Some of these alternative antioxidants will probably be discussed in subsequent papers

DR MOORE I should like to ask whether there is any close similarity between the relationship of vitamin E deficiency and potassium deficiency

DR LING I should like to mention one report by Dr Martin, who is also

one of Dr McCollum s students, in connection with the vitamin E deficiency Dr Martin published a paper in 1946 describing the chronic vitamin E deficiency produced in male rats In the castrated male rats the muscular dystrophy does not occur although the rats are on the same diet as other male rats I would like to ask Dr Mackenzie if he can explain the differences in response due to the influence of the male sex hormones in vitamin E deficiency

DR PAPPENHEIMER Can you answer that, Dr Mackenzie?

DR MACKENZIE No, I can't

DR PAPPENHEIMER It is interesting in this connection that castration in female rats is supposed to inhibit the deposition of the uterine pigments

VITAMIN E IN EARLY LIFE *

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INTRODUCTION

ALTHOUGH our topic for discussion pertains to vitamin E in early life, it may be pertinent to reflect for a moment on the early life of vitamin E My first acquaintance with our subject began almost 30 years ago, when it was experiencing a quiet intrauterine existence A few years later this young upstart was born into the vitamin family under a halo of questionable legitimacy at a time when the 4 previous children, A, B, C and D seemed, to many, to be sufficient to preserve man and lower animals from complete oblivion Subsequently reared under a veil of stigma and suspicion, the post adolescent individual is now verging on a state of schizophrenia—extolled by some as a supreme panacea of many human ills, regarded by others as the elixir of a charlatan, and subjected to public ridicule in lay magazines and on editorial pages of reputable scientific journals

Many vitamins have bridged with considerable speed and equanimity that hazardous gap between experimental exploration and practical application in clinical medicine Often this has occurred despite the fact that experimental exploration was in an immature and controversial state This has been the case with the 3 fat soluble vitamins, A, D and K However, the story of vitamin E (or alpha tocopherol) is quite different

On the experimental side, all species of animals depleted of vitamin E, ranging from the guppy fish to the monkey, have exhibited

* Acknowledgment is made to the Nutrition Foundation Inc the National Vitamin Foundation Inc the Masonic Foundation for Research and Human Welfare and the Helen Hay Whitney Foundation for generous support of the studies which are summarized in this lecture

certain deficiency manifestations Experience with farm animals, either intentionally or unwittingly deprived of vitamin E, have resulted in similar dysfunctions Lesions of skeletal muscles are essentially universal findings Effects on smooth muscle, cardiac muscle, reproductive systems and vascular system show considerable species variation Likewise, the level of tocopherol in the blood plasma, milk, and body tissues and organs of animals varies within wide limits Yet, no one denies that vitamin E has important functions in the metabolic economy of lower and higher vertebrates What the specific function, or functions, may be still remains a mystery, but there is considerable evidence that tocopherols exert some regulatory control over intracellular oxidations through yet unknown mechanisms

In man, as in the case of lower forms, tocopherols are widely distributed in tissues and organs, including the blood and milk, are transferred across the placenta to only a limited extent, and are poorly absorbed under conditions similar to those which limit the utilization of other fat soluble vitamins Unlike the case of the latter, a state of clinical or subclinical avitaminosis E has never been recognized in man Furthermore, those clinical conditions which have been reputed to benefit by tocopherol therapy are usually quite dissimilar to the dysfunctions experimentally induced, while the close counterparts of the latter in man are generally unresponsive to such therapeutic measures Perhaps the wide distribution of tocopherols in nature and their tenacious storage by the tissues, *chiefly the adipose tissue, preclude an outspoken deficiency state* If a suboptimal state of vitamin E nutrition does occur in man, either as a natural or conditioned depletion, clinical experiences relating to the other fat soluble vitamins would direct attention to the vitamin E status during prenatal and early postnatal life, before tissues have stored much of the vitamin In experimental studies this is also the period where deficiency manifestations are most readily induced and most striking

During recent years investigations in our laboratory have been directed along two major lines

1 A study of the diminished ability of young low E rats to

tolerate and survive certain types of dietary stress, and the influence of the latter in accelerating and accentuating manifestations of the deficiency state

2 A study of tocopherol distribution and storage during fetal life, infancy and adolescence in man, in the hope of formulating some picture of the normal status of vitamin E and the manner in which it may be altered by congenital or acquired dysfunctions

Although these two programs of research may appear to be rather distantly related, there are basic patterns common to both which may have increasing significance as the accumulation of additional data and new approaches help to dispell the investing haze To those who have contributed so enthusiastically to the planning and conduct of these studies—Dr Lloyd Filer, Jr , Dr Stanley Wright, Miss Ruth Rumery, Miss Mei Yu Dju and Dr Samuel Shaver—I wish to express the appreciation of one who sat chiefly on the sidelines We are also indebted to the departments of Pediatrics Obstetrics and Gynecology, and Pathology for their cooperation and assistance in these studies

Dietary Stress in the Rat

Returning briefly to the studies with rats, let us first recall the well known fact that in this species, maternal deficiency of vitamin E causes resorption of fetuses *in utero* or, if less severe, the occurrence of paralysis in the suckling young at 18 to 25 days of age Depending on the severity of E depletion the young rats showing late lactation paralysis either recover spontaneously or die within a few days

Some five years ago we noted (1) that 21 day old stock rats fed low E diets high in unsaturated fats, such as cod liver oil or methyl esters of linseed oil, frequently succumb between the fortieth and sixtieth days of life with symptoms similar to those seen in rats dying of late lactation paralysis If placed on the experimental diets at 30 days of age, good health is maintained throughout this period of adolescence Miss Rumery has since explored the effects of such diets at earlier ages, by rearing stock colony mothers and newborn litters on the low E diets high in unsaturated fats, beginning on the day of delivery It should be emphasized here that suckling rats

gradually acquire free eating habits only during the last third of the 3 week period of lactation, so that effects of the experimental diets during this period are due chiefly to the mammary transfer of metabolites A combination of 18 per cent lard and 2 per cent cod liver oil in the low E diet has no outward effect on the young rat When the dietary fat is 10 per cent cod liver oil, about 90 per cent of the infant rats show late lactation paralysis and none recover spontaneously When the cod liver oil is increased to 20 per cent all suckling rats die before the twenty first day of life, often with no characteristic paralysis The same diet fed to 21 day old stock rats results in a high incidence of death, sometimes associated with paralysis, between the fortieth and sixtieth days of life, when fed to 30 day rats good health is maintained to adulthood When the total fatty acid fraction of cod liver oil, equivalent to 20 per cent of the whole oil is incorporated in the low E diet and fed from birth, many of the rats at 20 to 30 days of age exhibit rather extensive hemorrhages, usually subcutaneous but sometimes pulmonary, which bear certain resemblances to the exudative diathesis and nutritional encephalomalacia characterizing the vitamin E deficiency state in chicks and known to be influenced by the nature and amount of dietary fats (2) In striking contrast, tocopherol supplements enable the young rat to tolerate with ease the dietary or metabolic stresses associated with high intake of unsaturated fats

Two recent reports (3, 4) present evidence that steatitis, or yellow fat disease in mink, responsible for severe economic losses *in the mink industry, is a true counterpart of the syndrome in young* rats just described It occurs only during the early post weaning period, is characterized by the same striking histologic alterations of adipose tissue, is associated with inadequate vitamin E and high intake of highly unsaturated fats (particularly frozen fish scrap), and can be prevented by tocopherol therapy

The protective role of vitamin E in early life is also illustrated by another series of studies (5) primarily designed to verify reports that prolonged feeding of silver nitrate to rats produces argyrophilia of reticular fibers of connective tissue Weanling rats reared on laboratory chow and drinking water containing 0 15 per cent of

silver nitrate maintain good health for 18 months or more Litter mates reared in like manner on a low E diet invariably die during the second or third month of life, despite the fact that water intake, and consequently silver intake, is much less on the synthetic than on the laboratory chow diet As in the case of unsaturated fatty acid feeding, respiration and body temperature diminish, a gradual onset of lethargy and coma precedes exitus, and neither gross nor histologic findings provide a reasonable explanation of death When rats are placed on experiment at 30 days of age, mortality is still high but certain animals survive and continue for prolonged periods with no fatalities Yet supplementation of the 21 day old animals with tocopherols during the experimental period, or priming of animals with tocopherols before silver nitrate feeding is initiated, provides complete protection against the detrimental effects of the silver Since other silver compounds reacted in similar manner, this cannot be construed as a nitrate poisoning

These are but two examples of the efficacy of tocopherols in enabling the young rat to overcome dietary or metabolic stresses at two critical periods of early life (1) during the late lactation, or weaning, period, and (2) during the adolescent, prepubertal period of life There are other examples of tocopherol protection against the effects of anoxia (6)—low protein diet (7, 8), carbon tetra chloride poisoning (9), hepatic necrosis (10, 11, 12, 13), and other stresses in adult rats—and against hemolyzing effects of alloxan decomposition products in the young rat (14, 15) The last phenome non, and possibly others, will be discussed by Dr Gyorgy I trust that he shares, to some degree at least, the general viewpoint that I have emphasized

Vitamin E in Man

Let us consider now inquiries into vitamin E and early life in man The initial approach has been to learn as much as possible about the deposition and storage of tocopherols in human tissues and organs, beginning with early fetal life Despite the laboriousness of the method for the chemical determination of tissue tocopherols, and certain difficulties in securing the materials desired for these

analyses, Miss Dju has made a notable contribution in an area where but little factual knowledge previously existed It was first estab lished that under usual conditions of post mortem refrigeration and subsequent deep freeze storage of tissues, for periods up to 3 weeks, the loss of tocopherol is relatively slight (16) What I present to you today is little more than a progress report on a series of analyses carried out on tissues obtained in the Rochester area, supplemented by material shipped in from outside sources with the assistance of dry ice, thermos bags and air transport To simplify the data as much as possible, I have summarized them in a series of graphs, which follow

Placentae and Total Fetuses (3 6 months)

The first graph summarizes analyses of entire fetuses, and of placentae, at 3 to 6 months gestation age (Fig 1) Most of these represent therapeutic abortions The consistently higher tocopherol

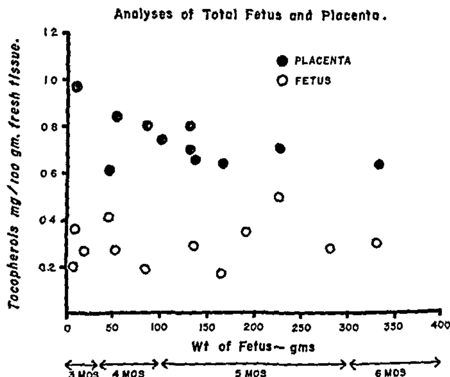


FIG 1 Tocopherol content of 12 human fetuses of 3 to 6 months gestation age and of 11 placentae in 8 instances fetuses with corresponding placentae are represented

evel in the placenta compared to the fetus is apparently a real difference between tissues, and not one due to placental retention of maternal blood which normally shows a considerable increment in tocopherols (from about 1 2 to 1 9 mg per cent) during pregnancy

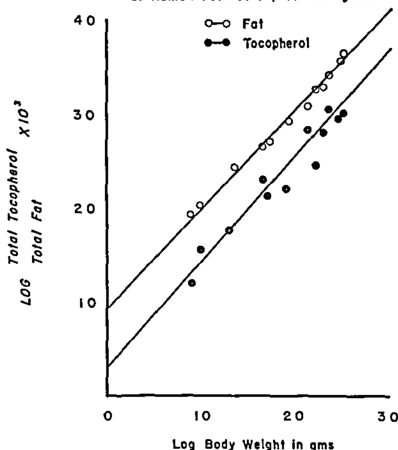


FIG 2 Interrelationship between total fat content and total tocopherol of 12 human fetuses The calculated slope of each curve is essentially unity

This higher placental level is in accord with evidence from analyses of fetal and maternal blood in man (17, 18) and from animal experiments (19) demonstrating that placental transfer of tocopherols is decidely limited and influenced but little by large increases in the maternal intake Note also the relatively small variations

in both placental and fetal levels of tocopherol, and lack of increment in these levels as gestation progresses The placental values compare closely to those reported by Athanassiu (18, 20) There also appears to be an interesting, and possibly significant, relationship between the fat content and tocopherol content of the fetuses when these are plotted against fetal body weight on a log log basis (Fig 2)

Tissues—fetus, premature, newborn

In the third graph (Fig 3) are portrayed minimal, maximal and mean tocopherol levels for

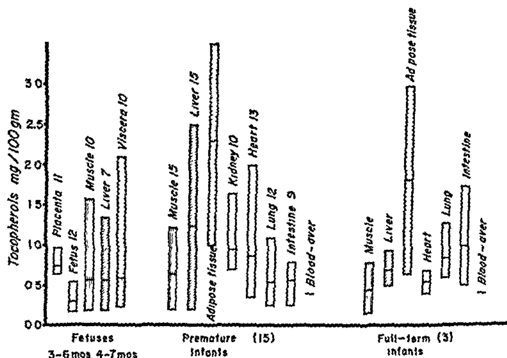


Fig 3 Showing the minimum maximum and mean tocopherol content of 3 4 month fetuses and placentae recorded in figure 1 and of various tissues and organs of 4 month fetuses and of premature and full term infants dying within a few days after birth The figures indicate the number of subjects from which samples for analysis were obtained

(1) the 11 placentae and 12 fetuses just discussed

(2) muscle, liver and viscera of 10 other fetuses

(3) a larger variety of tissues and organs from 15 premature and 3 term infants, which succumbed within a day or two after

birth Since atelectasis or congenital defects of heart and central nervous system were the chief pathologic findings, they presumably represent reasonably normal subjects from the standpoint of tocopherol levels

It will be noted that mean tocopherol values for muscle and viscera, and for kidney, heart, lung and intestine separately, while slightly higher than for total fetuses at 3 to 6 months, maintain low levels of between 0 5 and 1 0 mg per cent in all 3 groups, the somewhat higher mean level for the liver in prematures may be of significance, but the low liver value in full term infants is probably not representative However, these tissue levels are invariably higher than the mean blood levels (about 0 4 mg per cent) in premature and term infants On the other hand, tocopherol levels in adipose tissue are significantly higher than those in any other tissues, and 5 to 6 times higher than blood levels The samples of adipose tissue analyzed always consisted of a mixture of subcutaneous and retro peritoneal fat

Fat deposition begins in fetuses of 800 to 1,000 grams (i e at 6 to 7 months of gestation), according to Widdowson and Spray (21) In premature infants it is histologically immature and glandu lar in type, and usually so sparse that it is difficult to secure a very generous sample for analysis At birth it is mostly embryonic in type but considerably more abundant in well developed infants This early acquisition of tocopherols by adipose tissue is of interest in view of the evidence that adipose tissue in adult man is the major site of deposition of tocopherols (22), although it does not serve as a storehouse for vitamin E in the way that the liver functions in the storage of vitamin A

Birth to Old Age

The data in the next graph (Fig 4) are fragmentary, exploratory, and largely suggestive of where future emphasis should be placed The primary objective in this phase of our study was to acquire information on the tocopherol content of tissues and organs at early ages in man, to provide a picture of the normal rate of acquisition and the pattern of distribution of tocopherols from birth to sexual maturity, and to provide a yardstick for evaluating analyses of tissues

obtained from individuals suffering from (biopsy of tissues) or succumbing to (autopsy tissues) certain types of disease That we have acquired more of the latter type than of the normal type of material, and even then at a rather slow rate, is related of course to the low mortality rate, and rarity of accidental deaths with subse-

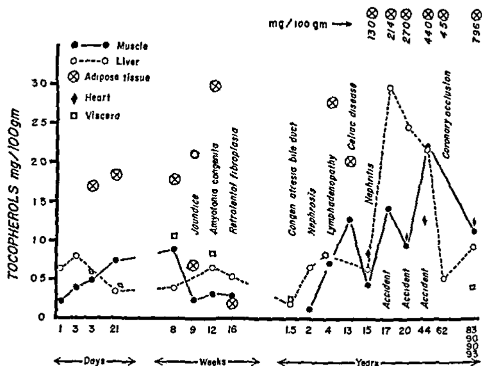


FIG 4 Tocopherol levels in tissues from 21 subjects, obtained at autopsy except in the case of celiac disease where biopsy was done The age of subjects is recorded on the abscissa The 5 subjects at the left, and 8 at the right, of the graph are considered normal The lines connecting symbols do no more than suggest certain trends

quent post mortem examination, in these age periods—especially in hospitals affiliated with medical schools While we have made our needs known to many other medical centers, and worked out details for deep freeze storage and air mail transport of tissues in the frozen state, the response has been discouragingly feeble to date The data on tissues from individuals of advanced age resulted from natural curiosity combined with opportunity to process such tissues while waiting for material representative of the early age groups

In the graph (Fig 4), directly above each age period on the

abscissa are tocopherol values for one or more of the tissues and organs indicated by the symbols listed at the upper left corner In some instances only certain of these tissues could be obtained To simplify the data presented, under the heading "viscera" are averages of values obtained on lungs, kidney, spleen or intestine, since individual values on these organs are generally of much the same order The lines connecting symbols for muscle values and liver values have no particular significance, but indicate general variations and trends The 5 cases shown at the extreme left, and the 8 at the extreme right represent what may be regarded as normal individuals for their age periods, the data on the 20 and 44 year accident cases have been taken from the analyses reported by Quaife and Dju (22) The remaining 8, covering age periods from 8 weeks to 15 years, represent a variety of disease states as recorded on the graph

Muscle tocopherol appears to increase progressively from birth to 8 weeks, although this may be coincidental Liver tocopherol does not show this trend In the 7 disease states which came to autopsy (the muscle and adipose tissue of the patient with celiac disease were obtained through biopsy), the muscle and liver values are consistently below 1 0 mg per cent, and as much above as below the values at birth Values for both tissues, the liver in particular, tend to be higher in the 3 cases of accidental death in adults, and to decline toward levels characterizing early postnatal life in the four individuals at 83 to 93 years of age It is interesting to note that tocopherols in heart muscle in the latter group, and in 3 other cases, are slightly greater than in skeletal muscle The high liver tocopherol level in the case of jaundice at 9 weeks is of interest, in that the fat content of this liver was also unusually high

Of greater interest are the tocopherol levels in adipose tissue At 3 days to 8 weeks these are quite similar to those for newborn infants shown in the previous graph In the cases of jaundice and retrolental fibroplasia the values are exceedingly low This is not so in the case of amyotonia congenita The adipose tissue levels in the case of lymphadenopathy and celiac disease, and particularly the latter, appear abnormally low on the basis of levels observed at the 15, 17, 20 and 44 year periods, which exceed by 4 to 15 times

the limitations of the ordinate of the graph By the same token, the five individuals representing the latter decades of life (62 to 93 years) show rather low levels of tocopherol in their adipose tissue

It is apparent that no far reaching conclusions can be derived from the data at present available However, there are indications that as many other pieces of the puzzle are located and assembled, especially those relating to the normal levels of tocopherols in tissues during infancy and childhood, and also in old age, findings relative to tocopherol distribution in certain clinical disorders will take on greater significance

Blood Versus Tissue Levels

Unfortunately, in studies of this type one is rarely able to obtain blood samples prior to death There is great need for data correlating blood and tissue tocopherol levels, if we are to determine what significance can be attached to differences in serum tocopherol values The development of a micromethod for serum tocopherols (23) makes it possible to carry out about 50 such measurements per day, as compared to a maximum average of 2 tissue tocopherol determinations per day But just what serum tocopherol levels represent, in terms of dietary intake and tissue storage of tocopherols is an open question at the moment

Tocopherols in Early Postnatal Life

Let us return now to early postnatal life, where serum levels appear to have more significance than in the adult As we have seen, in both premature and full term infants, tissue tocopherols are relatively low and not subject to particularly great variations from subject to subject, they presumably increase moderately during the last trimester of pregnancy What happens to tissue tocopherols during the first 2 months of postnatal life may, or may not, be correctly indicated by the fragmentary data in the previous graph (Fig 4) Serum tocopherol levels are also low, usually ranging from 0 3 to 0 4 mg per cent Furthermore, we have a general picture of what happens to them during the first six months of postnatal life (24, 25)

In the next graph (Fig 5), I have indicated very approximately

the trends in tocopherol increments in the fetus previously discussed, and in the maternal blood during pregnancy This maternal increase, observed by Straumfjord and Quaife (17), Scrimshaw *et al* (26),

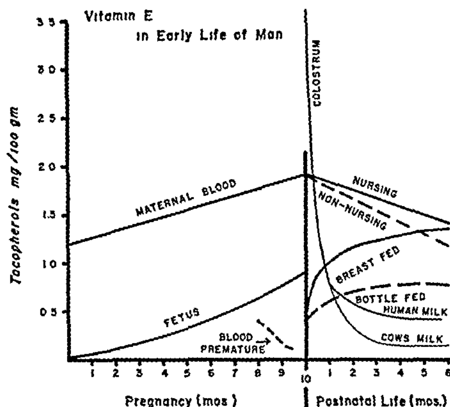


FIG 5 A somewhat schematic summary of tocopherol relationships during early life in man For further explanations see text

Darby *et al* (27) and others is a fascinating but perplexing phenomenon It may be prerequisite to passage of adequate tocopherols across the placental barrier, it may reflect a relatively greater utilization of tocopherol by the fetus than by the mother, though this seems unlikely, it may represent a physiological preparation for tocopherol transfer to the colostrum to compensate for restricted placental transfer and to better protect the infant against the exigencies of early extrauterine existence The evidence at hand would favor the latter interpretation

The studies of Quaife (28) and of Abderhalden (29) indicate

that during the first week or two *post partum*, human milk may often have a tocopherol content of 3 0 to 3 6 mg per cent, while later milk contains only about one sixth this amount Similar differences between colostrum and later milk have been reported for the cow, sheep, goat and pig In Fig 5, the fine line curve, which should have a steeper slope to be more accurate, represents this marked drop in tocopherol content of milk, and illustrates differences in the plateau levels for human and cows' milk, as estimated from various reports in the literature

The other lines and curves attempt to summarize results recently reported from our laboratory (24) In striking contrast to the high maternal serum levels of tocopherol, term infants on the first or second day had a mean serum level (0 3 mg per cent) approximately one fifth that of the mothers Those which were breast fed showed a rapid increase in serum tocopherols, while maternal blood levels declined more slowly than in non lactating mothers The latter suggests that maintenance of lactation, as well as the preparation for it, necessitates a high level of blood tocopherol In non nursed infants, on the other hand, mean serum tocopherols levels were much lower and showed a very slight increment, which is not surprising when one considers that cows milk possesses less than one fifth the tocopherol content of human milk It was calculated that the artificial formula used in these studies contained approximately 0 1 mg tocopherols per 100 cc

The premature infants, while starting life with essentially the same serum tocopherol level as the term infant, received a diluted cows milk formula, because of poorer tolerance of the premature for fats It will be noted that serum tocopherols declined rather rapidly reaching a level of 0 09 mg per cent at 31 to 40 days of life This fall in serum levels may represent the effect of several unrelated factors (1) inadequate supply of tocopherol, with an artificial formula providing about 0 04 mg per 100 cc, (2) an excessive demand for tocopherol to meet the metabolic stresses related to the precocious extrauterine environment, or (3) a possible redistribution of tocopherol due to an increase of blood volume and hemodilution In any case, we observe in these premature arti

fically fed infants serum tocopherol levels closely approaching those which in the experimental animal are associated with the appearance of deficiency symptoms Whether the maintenance of a critically low level of serum tocopherol for a considerable period of time, before the institution of a mixed diet, can exert a harmful effect or diminish the capacity of the infant to overcome metabolic or other exigencies, remains to be ascertained

A current report (25) presents evidence that premature infants can absorb and presumably retain tocopherols, free or esterified, as effectively as can the full term infant, which stands in contrast to the reported lesser effectiveness of absorption and retention of vitamin A (as oil) and of fats in general in premature as compared to full terms infants This was demonstrated by tolerance or response curves based on blood samples obtained at 3, 6 9, 12 and 24 hours after a standard oral dose (20 mg tocopherol per kg body weight) and evaluated on the basis of planimeter measurements of the area under the curve

A series of tolerance tests on 19 older infants suggested an appre ciably reduced absorption in cases of diarrhea, biliary obstruction, pancreatic fibrosis cirrhosis and some types of malnutrition There was a striking improvement in absorption in a patient with fibro-cystic disease following active therapy with pancreatin In another group of children ranging from 2 to 16 years of age, lupus, fibro cystic disease and the celiac syndrome were characterized by flat response curves On the other hand unusually high response peaks were observed in metabolic disturbances such as diabetes and nephro sis Such responses are possibly related to the associated hyperchole steremia, which is often associated with increased serum tocopherol levels (27)

In general the low tocopherol response curves observed in diseases of children are in accord with findings reported by others in adults with primary fibrositis, cirrhosis and obstructive jaundice (30 31, 32) As we have seen similar diseases appear to be associated with low tissue levels in children (Fig 4) It is not clear to what extent these phenomena result from defective absorption and to what extent they reflect metabolic dysfunctions influencing the fate of tocopherol once it has passed the intestinal barrier

Conclusion

Our discussion has touched upon many points, some of which may not seem too closely related to each other, let me recall (1) the capacity of vitamin E to combat metabolic stress in the rat, (2) the limited placental transfer and more generous mammary transfer of the vitamin in man, phenomena which are common also to laboratory and farm animals, (3) the contrasting plasma tocopherol levels between bottle fed and breast-fed infants, and especially the low plasma levels in the artificially fed premature infant, (4) the function of adipose tissue as a reservoir, but not a true storage depot, for tocopherols, (5) and the fragmentary suggestions that metabolic and other diseases of childhood may be associated with low tissue levels of tocopherol and with impaired absorption of dietary tocopherols

I have attempted in Fig 6, to simplify and summarize certain aspects of this general picture of vitamin E in the early human life, as it appears on the basis of data at present available If we assign the value of 1 to tocopherol concentration in fetal blood (based upon average levels of about 0 40 mg per cent observed in newborn infants), it appears that fetal tissues and the placenta would have values of 1 to 2, whereas those of maternal blood and maternal tissues would have values of 5 and 10 to 12, respectively, the latter figure is based on estimates of total tocopherols in 2 adults (22, 33) The tocopherol content of the lactating mammary gland is unknown, but colostrum milk may have almost twice the tocopherol concen tration and later milk about one third the concentration, of maternal blood The breast fed infant benefits promptly from this provision of nature, attaining blood levels above those of the adult at the end of the first week of life, the bottle fed infant has appreciably lower bloods levels than the breast fed infant even 6 to 8 months after birth The bottle fed premature has levels sometimes not measurable by the microchemical method With the exception of adipose tissue, the tissue tocopherol levels of the premature and full term infant at or shortly after birth are quite comparable to those of the late fetus Our real bottleneck lies in lack of material on which to

establish a picture of the normal increment in tissue tocopherols during infancy, early childhood and adolescence, against which may be assessed data such as we have begun to acquire on clinical disorders of early life

Late fetus
and Newborn

Blood Tissues
1 1 2

Early infancy
(2 8 weeks)

Blood Tissues

Childhood
and Adolescence

Blood Tissues

Breast fed 3 1 3
Bottle fed
premature < 1 < 3 3 >>

Placental Barrier
2

early MILK later
5 10 0 5 1
Mammary Barrier
>

Maternal Blood
(late pregnancy and early lactation)
5

Normal Adult
Blood 3 Body as a whole 10 12

FIG 6 Tocopherols in man A schematic summary illustrating approximate estimates of the relative tocopherol concentrations in man (in terms of mg tocopherol per 100 gm fresh tissue) accepting as unity the tocopherol concentration in the blood of the newborn infant (approximately 0 4 mg per cent) For further explanation see text

Already questions arise to which we need answers To what extent may tissue storage be diminished by or even be a factor in lessening resistance to, certain metabolic disease states during early postnatal life? Should the pediatrician take heed of the picture already revealed in the bottle fed infant especially the premature infant? If he believes in the fundamental principles of preventive medicine, I am convinced that he should and eventually will What is the vitamin E status at the other extreme of the life span? Will more adequate information of this type provide some clue to the functions of vitamin E in man? I feel certain that it will *

* Since the preparation of this paper our analyses of tocopherols have been considerably extended and the results which generally confirm the observations recorded here have been published in part (34) and recorded in greater detail in thesis form (35)

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SOME METABOLIC EFFECTS OF VITAMIN E

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IT IS A VERY great honor for me to participate in the Symposium honoring our Master, Dr McCollum, to whom nutritional research and we, its soldiers, owe so much For me, this occasion is a special distinction for strictly personal reasons Dr McCollum, Dr Pappenheimer, and Dr Park, among those present today, have, without their knowledge, exerted a decisive influence on my own scientific development and life about 30 years ago I tried to duplicate their work carried out in collaboration with their colleagues, such as Alfred Hess, Shipley, Simmonds, on experimental rickets For reasons still unknown, my efforts were unsuccessful Youth, combined with inexperience led me then to a very unwholesome critical attitude regarding animal experiments and their application to clinical medicine It did not take longer than 1 to 2 years to learn my lesson Since that time, I have rather overcompensated for my original scepticism and in all my scientific effort I prefer to rely on exact experimental foundations This I owe Dr McCollum and his colleagues

Further, it is a special pleasure for me to appear on the same program and with the same purpose—honoring Dr McCollum—with my most generous friend Dr Park, whose continuous good will and help paved my way in this country

Today we are discussing one step child of vitamin research vitamin E In the first part of my presentation I wish to elaborate on the role of vitamin E and on the rather complicated interrelation of vitamin E with other dietary and similar factors in dietary hepatic injury The remarkable advance in our knowledge of hepatic injury achieved during the last 10 to 12 years offers a good illustration of the value and role of the experimental approach

From a pathologic point of view necrosis and cirrhosis are the characteristic manifestations of injury to the hepatic parenchyma Fat infiltration per se is not specific enough and is often too transient, without concomitant manifestations of tissue reaction, to be considered in itself as a truly pathologic manifestation of the liver

The experimental production of hepatic injury is by no means a recent achievement The literature dealing with such efforts and attempts is not only voluminous but antedates by several decades the developments of recent years

Four outstanding results characterize the latest developments in the experimental approach to diseases of the liver (a) the recognition of purely nutritional factors as important determining causes of hepatic injury, (b) the prevention arrest, and even possible reversal of this pathologic process simply by proper change in the composition of the experimental diet, and (c) the interrelation between dietary and endocrine factors in the pathogenesis of hepatic injury The fourth result embodies the concept that experimental dietary injury to the liver is often combined in the same animal with specific manifestations in the kidney This simultaneous occurrence of changes in the liver and kidney may have a bearing on the widely contested concept of the so called hepato renal syndrome

Dietary factors determining experimental liver injury in rats are summarized in the following table

TABLE 1

DIETARY FACTORS IN LIVER INJURY

	Cirrhosis	*Necrosis*
Protein	Beneficial	Beneficial
Methionine	Beneficial	Beneficial
Cystine	Injurious	Beneficial
Choline	Beneficial	No effect or injurious
Vitamin E	No effect	Beneficial
Dietary Fat	Injurious	No effect or injurious
Vitamin B_{12}	Beneficial	No effect

The dietary factors beneficial in the prevention of cirrhosis as the more chronic form of hepatic injury may be identified by one common denominator i e, by a sufficient supply of lipotropic factors in

particular of choline and its precursors The benefit seen after administration of vitamin B_{12} may be due to its choline sparing effect

In the acute form of experimental dietary hepatic injury, characterized by massive, often hemorrhagic necrosis, the beneficial dietary factors are cystine and methionine or vitamin E In contrast to cirrhosis, it is difficult to reconcile pure deficiency as the possible cause of dietary hepatic necrosis with the interchangeability of substances chemically as different as the sulfur containing amino acids, cystine or methionine, and the fat soluble vitamin E in the prevention of hepatic necrosis The assumption has been made that the beneficial effect of the sulfur containing amino acids, cystine or methionine and tocopherol, more probably results from an underlying detoxifying mechanism than from the supply of a deficient nutritional factor Both vitamin E and the sulfur containing amino acids are known detoxifying agents which may counteract the noxious effect of hepatotoxic and related substances In the case of dietary hepatic necrosis such toxic substances may originate in the intermediary metabolism or under the influence of the intestinal flora, particularly in the large intestine

Our first approach was directed toward the elimination—or at least the modification—of the intestinal flora as a possible source of factors injurious to the liver It has been shown that aureomycin, when added to the necrogenic experimental basal diet, containing yeast as the sole source of protein, had a significantly beneficial effect in the prevention of experimental hepatic necrosis in rats In contrast to vitamin E or the sulfur containing amino acids, cystine or methionine which as supplements to the basal experimental diet will permanently prevent the production of hepatic necrosis, aureomycin was found to delay as a rule the appearance of necrosis, and thus its protective action was only temporary

In analyzing this effect of aureomycin it should be pointed out that intensive studies have revealed no indication of an underlying systemic or focal hepatic infection as the direct cause of experimental dietary acute necrosis of the liver Thus the choice lies among (a) the supply of a missing antinecrogenic substance, (b) a direct metabolic and (c) an antimicrobial effect on the intestinal flora

If the effect of aureomycin is mediated by the suppression or modification of the intestinal flora, other antimicrobial agents should also prove to be effective, although not necessarily equal to aureomycin, depending on their bacterial spectrum and on the ease with which they may produce resistant strains Thus, it seemed advisable to study the effect of various antimicrobial agents on the production of dietary hepatic necrosis, especially in comparison with the effect of aureomycin

Polymyxin, chloromycetin and bacitracin have been found ineffective, sulfaguanidine and streptomycin slightly effective Neomycin, and even more terramycin, organic base salts of penicillin, in addition to aureomycin have shown higher activity

Ingested streptomycin is absorbed from the intestinal tract only in traces Thus, its beneficial effect further suggests suppression of the intestinal flora as the mode of action Observations with penicillin are in good accord with this assumption Whereas penicillin given by injection was either without any, or of only limited beneficial effect, penicillin given by mouth, especially its poorly soluble identical organic base salts, exerted very marked protection, not less than that of aureomycin

TABLE 2

Exp	*Date*	Survival time in days (av) *Control*	*Aureomycin*
1	November 1949	34	110
2	January 1950	41	103
3	August 1950	34	58
4	October 1950	32	42
5	January 1951	27	34

During the course of these investigations the delaying effect of aureomycin on experimental hepatic necrosis became gradually less pronounced with successive experiments carried out between November 1949 and March 1951 The results recorded permit the conclusion that the average survival time of rats kept under *identical* conditions and fed aureomycin in addition to the necrogenic diet has been considerably reduced, whereas in the same time, the survival of the control rats (not receiving aureomycin) has remained practically unchanged

During this whole experimental period the same strain of rat (Sprague Dawley) with the same initial weight and with the same experimental diet was used Batches of aureomycin from 1949 were compared with more recent batches and the same reduction in the survival time of the treated rats was obtained with "old and "new" aureomycin

In January 1951, when the aureomycin effect seemed to escape us, experiments with the identical strain of rats and the identical experimental diet (containing ingredients from a common source) were set up by us in two other laboratories in which similar experiments had never been conducted previously In both these new experiments the original long and very significant delay by aureomycin in the development of hepatic necrosis was again observed Further, the survival time of the control rats not receiving aureomycin was also significantly prolonged, compared with the figures given in Table 2 These results appear to be in accord with the assumption that in repeated experiments (a) aureomycin resistant bacterial strains and/or (b) an increase in virulence of an unknown agent, such as a virus, may have developed Increase in aureomycin resistance has bee demonstrated by us in rats receiving aureomycin supplement fo *E coli* and *Enterococcus*

The most impressive results were obtained in cooperation with Professor J A. Reyniers, T D Luckey and their associates at the Germ free Life Laboratory, University of Notre Dame Two limited experiments were set up with 6 to 8 rats as controls and 2 germ free rats in each of the two experiments The so called control rats received the same autoclaved necrogenic basal diet as the germ free animals but were kept in normal, non germ free laboratory surroundings All control rats died with massive hemorrhagic necrosis of the liver In contrast, the germ free animals lived twice as long as the experimental animals, and showed at autopsy no necrosis of the liver Their weight curve throughout the whole experiment was very satisfactory in contrast to the flat curve of the control animals It is of special interest that the germ free animals died with manifestations of severe hemorrhagic diathesis with consecutive anemia Pulmonary hemorrhage was a common feature, reminiscent

of acute experimental vitamin E deficiency Th
appears to be warranted that the germ free anim
necrogenic basal diet might have died from v
without involvement of the liver

These experiments are in further support of th
regarding the role of the intestinal flora in the p
hepatic necrosis However, the basic facts whic
the tri-cornered relationship between vitamin E, c
flora remain still obscure and require further e

In experimental dietary cirrhosis the appeara
fluorescent pigment called ceroid has been
be related to the brown pigment seen in vitamin
especially in the uterine musculature ovary or
from the fact that the fluorescence of ceroid is n
cal with that of the pigment occurring in simple
ceroid distinguishes itself also by the fact tha
prevented by vitamin E medication provided th
fat with a large proportion of unsaturated fatt
cod liver oil

More recently Hartroft has demonstrated that
ment of fatty cirrhosis fat may accumulate in the
extracellular fat cysts of various size The fat
eliminated through rupture of their wall either v
or may enter the sinusoids Through interaction
unsaturated fat ceroid develops *in situ* probably
tion The possibility that in these fat abscesses
be depleted by oxidation and—being extrace
easily replenished from dietary sources is by n

In the second part of this presentation we
effect of vitamin E deficiency on a specific form
blood cells These investigations were carried o
in cooperation with Dr Catharine S Rose

was followed by intravascular hemolysis of variable severity depen dent on the type of diet fed Mortality of the tocopherol deficient animals was very high during the first day or two after alloxan was given There was marked hemoglobinuria and at autopsy the kidneys were found to be engorged with blood This evidence of injury to the red blood cells was confirmed by hematocrit readings and blood counts, which indicated destruction of erythrocytes ranging from slight to 80 or 90 per cent beginning within ten minutes after the alloxan was injected Tocopherol gave complete protection There was no hemolysis in any of the scores of tocopherol treated animals tested

In these experiments the hemolyzing action of alloxan and the protective action of tocopherol were clear and unequivocal The mechanism of either effect was quite obscure Alloxan remains as such in the body for only a few minutes Under physiologic con ditions it is rapidly and irreversibly converted to alloxanic acid A portion of it is reduced to alloxantin and dialuric acid by means of such reducing agents as the sulfhydryl compounds The interrelation ships of these derivatives of alloxan, any one of which might be the actual hemolyzing agent, are illustrated in Fig 1

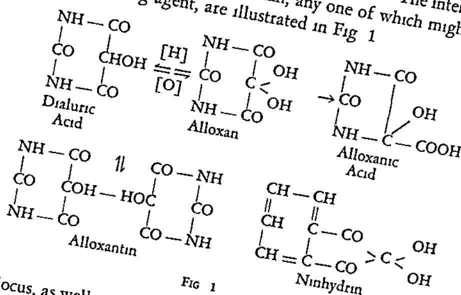


Fig 1

The locus, as well as the mode of action of tocopherol, was unknown It might react with alloxan or a related compound in the tissues, the blood plasma, or the red blood cell On the other hand, tocopherol,

as such, might not be involved directly in a reaction, but in its absence the erythrocyte might be defective in structure

Injection of compounds related to alloxan was the first means used to investigate the mechanism of its hemolyzing action These included, in addition to the alloxan derivatives which have been mentioned, ninhydrin This compound, as far as the triketone portion of the molecule is concerned, is a structural analogue of alloxan and undergoes similar reactions Since, with this method, specific effects on the blood cells could not be entirely separated from systemic effects, it was desirable to study the red blood cell *in vitro* A system was found in which hemolysis could be produced consistently, and direct studies were made of the alloxan like compounds and tocopherol The *in vitro* technic had the additional advantage of obviating the necessity of injecting the rat with alloxan, and repeated studies could be made on the same animal

The studies on erythrocytes *in vitro* have indicated that hemolysis is linked with dialuric acid rather than with alloxan

TABLE 3

HEMOLYSIS BY ALLOXAN LIKE CPDS IN TOCOPHEROL DEFICIENT RATS

	Vivo	*Vitro*
Alloxan	+	—
Alloxantin	+	++
Dialuric Acid	++	++++
Ninhydrin	++++	—

A similar mechanism *in vivo* is quite possible since at least a portion of injected alloxan is reduced in the body That dialuric acid administered to tocopherol deficient rats caused more severe hemolysis than did alloxan is in accord with this view On the other hand, it is difficult to formulate a reaction between tocopherol and dialuric acid to account for the inhibition of hemolysis in cells rich in vitamin E Under physiologic conditions alloxan and dialuric acid form a readily reversible oxidation reduction system Since tocopherol is an antioxidant, it is very probable that it reacts with some intermediate of the system, a free radical or peroxide, which is the actual hemolyzing agent

Ninhydrin, like alloxan in structure and reactions, had much greater hemolyzing action than alloxan *in vivo*. The analogue of dialuric acid, 1, 3 diketo 2 hydroxy hydrindene, could not be tested because its instability has prevented its isolation. There was no hemolysis *in vitro* with ninhydrin in the presence of reducing agents as there was with the corresponding alloxan system, probably becau[se] the instability of the reduction products of ninhydrin prevented the[ir] accumulation in sufficient concentration to react.

It is of interest that tocopherol deficient red blood cells could be hemolyzed by such normal metabolites as cysteine, glutathione, and ascorbic acid. Table 4 summarizes the results obtained with cysteine.

TABLE 4

HEMOLYSIS WITH CYSTEINE

Cysteine mM/L	Degree of Hemolysis Time (Hours)		
	3	7	18
5.2	—	—	++++
2.6	±	+	+++
1.3	+	++	Complete
0.65	—	—	++

The same mechanism may be proposed for the hemolytic action of these compounds as for that of dialuric acid, since they are all autoxi[-] dizable. Ascorbic acid and dehydro ascorbic acid, indeed, have the same structural relationship as dialuric acid and alloxan. It should, however, be emphasized that a rate of hemolysis equal to that with dialuric acid was never obtained with these compounds. It is a characteristic of hemolyzing agents that in higher concentrations they form a protective layer around the cell which inhibits hemolysis. The approximate equality of the hemolyzing and fixative concen[-] trations of cysteine, glutathione, and ascorbic acid may account for the very slow hemolysis with these substances.

The problem of determining whether tocopherol added directly [t]o a suspension of red blood cells, would protect them as it did [i]n *vivo* was complicated by the fact that tocopherol is not water [so]luble. We have overcome the difficulty by using Tween 80 as

dispersing agent If present in large amounts Tween would interfere with the determinations, since it is in itself a hemolyzing agent However, tocopherol proved to be so effective in preventing hemolysis that the amount of Tween present was never more than one tenth of the minimum amount which would cause any acceleration of the rate of hemolysis by dialuric acid

First, tocopherol was added to the red cell suspension just before the dialuric acid (0 05 to 0 2 mg per cc) On the basis of several tests, the minimum concentration of tocopherol which would give complete protection was about 0 01 mg On a molar basis 1 mol of tocopherol was inhibiting 30 or 75 mols of dialuric acid

TABLE 5

HEMOLYSIS PREVENTION BY TOCOPHEROL *in Vitro*

RBC, 2 5% *Procedure*	Dialuric Acid 0 7 mM/l *Tocopherol*
	mM/l
Toc added with D A	0 009
RBC incubated with Toc	0 0009
RBC in serum incubated with Toc	> 0 012

It may be assumed that in tocopherol treated animals the protective action is within the cell When tocopherol was added to the mixture of deficient cells and dialuric acid, the dialuric acid might be inactivated in the solution before it had an opportunity to come in contact with the cells To determine whether this was the case or whether the erythrocytes adsorbed the tocopherol or the tocopherol in some manner changed the surface layer of the erythrocytes, cells were incubated with tocopherol for 30 minutes at 37° C At the end of this time the tubes were centrifuged, the supernatant fluid was removed, and the cells were resuspended in saline and treated with dialuric acid in the usual fashion With this procedure the effectiveness of tocopherol was increased ten fold

The red blood cells of tocopherol deficient animals suspended in plasma or serum reacted as they had in buffer solution dialuric

acid was hemolytic, alloxan was not When tocopherol was added to the mixture of plasma (serum) and red blood cells, it appeared to combine preferentially with plasma (or serum) It required about 10 to 30 times as much tocopherol to protect red cells suspended in serum as those in buffer solution

It may be assumed that tocopherol and protein factors of the serum (or plasma) could form lipo protein compounds in which tocopherol is in a biologically inactive, inert form The analogy to protein bound calcium and free, ionized calcium presents itself In further consequence total tocopherol values should not necessarily be identified with the biologically available tocopherol In this respect the hemolysis test may have its practical significance

Susceptibility to hemolysis by dialuric acid differs from other manifestations of vitamin E deficiency in the rat in the short time required for its demonstration in relatively mature animals Sig of deficiency in the blood cells could be detected in rats 2 to 3 month old after only a few days on the tocopherol deficient diet, and th maximum rate of hemolysis was reached within 2 weeks Approxi mately 3 mg of DL alpha tocopherol per kg of body weight per day was required to protect the red cells This is several times the average minimum prophylactic dose for prevention of sterility or muscular dystrophy

In comparing the animals receiving the stock ration with those given supplements of tocopherol or a tocopherol deficient diet, it is disturbing to see how close these presumably normal animals were to the deficient level

This is apparent not only from the depletion studies but also from the plasma tocopherol values In rats receiving tocopherol supplement to their stock diet the average value of plasma tocopherol was found to be about 1 mg per cent The plasma of the rats receiving the stock ration contained less than half this amount of tocopherol, 0 38 mg per cent, while the level in the group on the deficient diet, 0 27 mg per cent, was not a great deal lower than that of the stock animals

Transfer of tocopherol across the placenta is known to be poor,

and it was of interest to determine the response of the erythrocytes of newborn rats to dialuric acid It was found strongly positive in newborn rats born of females kept on the usual stock diet In contrast, the newborn rats born of females receiving supplements of toco pherol (5 mg a day) showed complete protection of the red blood cells If, as these findings suggest, tocopherol serves as a protective agent against damage to erythrocytes (and perhaps other tissues) by toxic substances, such as dialuric acid, the fetus is in a peculiarly defenseless position, and may be damaged by conditions which would not affect the mother

In considering the physiological significance of the antihemolytic action of alpha tocopherol it was important to determine whether the protective effect could be demonstrated with other antioxidants or was a specific function of vitamin E Beta , gamma , and delta tocopherols should be of value in answering this question since, as antioxidants, they may be even more effective than alpha tocopherol, whereas the vitamin E potency of gamma and delta tocopherols as measured by fetal resorption test is negligible, while that of beta tocopherol is no more than one third that of alpha tocopherol In addition to the tocopherols a series of substituted hydroquinones and two estrogenic hormones were studied These substances, like the tocopherols, are fat soluble antioxidants

In studying the protective effect of the antioxidants 3 types of experiments were performed (1) incubation of vitamin E deficient erythrocytes with the protective agent *in vitro* followed by the hemolysis test with dialuric acid, (2) administration of the protective agent to the rat followed by the hemolysis test on the erythrocytes with dialuric acid, *in vitro*, (3) administration of the protective agent to the rat followed by injection of alloxan or dialuric acid

The compounds studied were the tocopherols, hydroquinone (for comparison with the fat soluble hydroquinones), benzyhydroquinone, di sec amylhydroquinone, sec octylhydroquinone, di sec octylhydro quinone, di iso octylhydroquinone, diethylstilbestrol and ethinyl estradiol

Tocopherols

In vitro, D, L alpha tocopherol at a concentration of less than about 0 3 gamma per ml did not protect red cells against hemolysis by dialuric acid, while with a concentration of approximately 1 5 gamma there was complete protection D Alpha tocopherol showed the same activity as D, L-alpha tocopherol D Beta , D gamma and D delta toco pherols were less active being respectively 0 4, 0 3, and 0 2 as active as D, L alpha tocopherol

TABLE 6

THE RELATIVE POTENCIES OF THE TOCOPHEROLS IN THE PREVENTION OF HEMOLYSIS BY DIALURIC ACID

Tocopherol	Mode of Administration Vitro	Vivo	Antioxidant Potencies * (against beta carotene at 39°)
DL Alpha	1	1	
D Alpha	1	1 4	1
D Beta	0 4	0 1	1 4
D Gamma	0 3	0 04	1 5
D Delta	0 2	0 03	1 9

* Stern *et al*, *J Am Chem Soc* 69, 869 (1947)

When D, L-alpha tocopherol was given orally to rats on the vitamin E deficient diet, the blood cells of animals receiving 0 4 mg or more per day remained completely resistant to the hemolyzing effect of dialuric acid If the curative procedure was used, a single dose of 1 5 mg of D, L alpha tocopherol restored the resistance of red cells which had been depleted of vitamin E The effect of the dose per sisted for several days A dose of 0 5 mg gave partial protection for one day *In vivo* the superiority of alpha tocopherol over beta , gamma , and delta tocopherols was more marked than it was *in vitro*, and a slight advantage of D over D, L alpha tocopherol was apparent Analysis of the protective study indicated that if D, L alpha toco pherol was assigned a value of 1 0, the activity of D alpha tocopherol was 1 4 while that of D gamma tocopherol was 0 04 D Beta toco pherol, with 15 animals treated, had an activity of 15 to 20 per cent that of D, L alpha tocopherol Because of the high dosages required a protective effect of D delta tocopherol was demonstrated in only

two animals which received 45 mg. One of these rats was completely protected for one day, the other only partially protected. This indicates an activity of no more than 3 per cent that of D,L alpha tocopherol.

Substituted hydroquinones

Di sec octylhydroquinone and di iso octylhydroquinone when tested *in vitro*, were almost as effective against the hemolyzing action of dialuric acid as alpha tocopherol, their activity was about twice that of beta tocopherol. Di sec amylhydroquinone and sec octylhyroquinone were less active but still had a marked protective effect. Benzylhydroquinone was inactive up to a level of 37.5 gamma per ml which indicates an activity less than 2 per cent that of alpha tocopherol.

TABLE 7

THE POTENCY OF VARIOUS ANTIOXIDANTS IN THE PREVENTION OF HEMOLYSIS BY DIALURIC ACID *in Vitro*

DL Alpha tocopherol	1
Di sec octylhydroquinone	0.8
Di iso octylhydroquinone	0.7
Sec-octylhydroquinone	0.2
Di sec amylhydroquinone	0.4
Benzylhydroquinone	0.02
Diethylstilbestrol	0.07
Ethinyl estradiol	0.03

Results with higher levels of benyzlhydroquinone were invalid because there was enough Tween 80 in the more concentrated solutions to cause hemolysis. Hydroquinone itself showed no protective effect up to a level of 100 gamma per ml. The 4 substituted hydroquinones which were active *in vitro* were tested *in vivo*: no protective effect could be detected.

Estrogens

Diethylstilbestrol and ethinyl estradiol had low but definite protective effect *in vitro*, the two compounds being respectively 7 and 3 per cent as active as alpha tocopherol. Only diethylstilbestrol was tested *in vivo* and found inactive.

acting as an enzyme was shown by the ineffectiveness of heated catalase

TABLE 10

THE EFFECT OF CATALASE ON THE HEMOLYSIS OF VITAMIN E DEFICIENT RED BLOOD CELLS BY DIALURIC ACID

Amount of catalase (ml per tube)	Hemolysis (%) Unheated catalase	Heated catalase
0	100	
0 01	99	100
0 02	84	100
0 05	22	75
0 10	0	92

Conc of catalase solution = 26μM ± 3μM

In summary, these more recent experiments indicate that there is a specific relationship between the vitamin E activity of a substance and its ability to protect the blood cells of rats deficient in vitamin E from the hemolyzing action of dialuric acid, when the substance is administered to the rat *in vivo* and the erythrocytes are tested *in vitro* Under these conditions, of the antioxidants tested, only the toco pherols showed activity Alpha tocopherol was more active than beta , gamma and delta tocopherols, and D alpha tocopherol some what more effective than D, L alpha tocopherol The values were similar to and in the same order as those reported for the relative activities of the tocopherols in preventing fetal resorption in rats

With respect to alpha tocopherol, Harris and Ludwig found the natural product 1 36 times as active as the synthetic form Our figure of 1 3 is in good agreement with this value

When the erythrocytes were treated with the protective agents *in vitro*, the protective effect was not limited to the tocopherols but was shown by the estrogens and by four of the substituted hydro quinones tested The latter group of compounds was very active, and the activity was proportional to the complexity of the substi tution, di iso octyl and di sec octylhydroquinones being more active than sec octyl and di sec amylhydroquinones while benzylhydro quinone and unsubstituted hydroquinone were inactive Beta , gamma and delta tocopherols were *in vitro* more effective in com

parison with alpha tocopherol than when they were fed to th
The ratios were, however, in better agreement with physiol
than with the antioxidant potencies of the tocopherols Two
substituted hydroquinones were more active *in vitro* than any
tocopherols except alpha tocopherol This was surprising since
structural similarity to the tocopherols is slight, whereas the
pherols themselves differ by only one or two methyl groups
difference between activity *in vitro* and *in vivo* may be due, in
to poor absorption from the intestine or to rapid alteration
compounds in the body interfering with their fixation on th
blood cells With the proven specificity for the living animal
the hemolysis test appears to be well suited for bioassay of vitan
... action of dialuric acid by catalas
... there

It does not prove that hydrog...
Removal of hydrogen peroxide from the solution might chan
rate of reaction or alter the concentration of some other interm
compound This view is supported by the fact that the concent
of hydrogen peroxide used in the tests was 0 4 M that of d
acid 0 001 M In spite of this difference hemolysis of E de
cells *was faster with dialuric acid than with peroxide That th*
cells of human adults and of rats treated with vitamin E are s
tible to hemolysis by hydrogen peroxide but not by dialuri
may be explained by the very short half life of dialuric ac
higher concentration of dialuric acid might overcome the pro
effect of tocopherol but the concentration of dialuric acid
shows the fixative effect characteristic for many hemolyzing
(including H_2O_2) is very little higher than the concentration
in the test

Experimental vitamin E research is one of the rare exce
regarding the applicability of animal studies to clinical med
It suffices, to refer to the unsatisfactory results with vitan
therapy in muscular dystrophy or in cardio vascular conditions
proven existence of vitamin E deficiency as demonstrable wi
hemolysis test, may open the way to a new approach in vita
therapy in conditions occurring during fetal or neo-natal life

Dr Quaife I ll be very brief I think that there are at least five people in the audience who can contribute information on this phase, and maybe I can be the one to add it I think that all of us who have worked with vitamin E want to know if alpha tocopherol carried in the blood is part of a protein complex Analyses for the amounts of various tocopherols occurring in the body show that alpha tocopherol is stored far more readily than beta or gamma or delta This is true for blood levels, for tissue storage in the body, for excretion into the milk or for laying down of the tocopherols in hens eggs I think Dr Wald s finding that the stereochemical forms of vitamin A show great difference in the protein combining powers is the answer If his suggestion is right, there must be a plasma protein which is highly specific for alpha tocopherol, not for beta, gamma, or delta Not only that, but I think that there s going to be one which is more specific for a particular stereoisomer of alpha tocopherol

Dr Mackenzie I should like to mention some experimental results obtained by my wife which are pertinent to the discussion When rabbits were placed on diet 11 of Goettsch and Pappenheimer, tocopherol could not be detected in the blood after one week Nevertheless, these animals did not develop a creatinuria or muscle lesion in an experimental period of three months In other words vitamin E deficiency, with respect to muscle lesions, did not exist even when the serum contained less than 0 1 mg per cent of tocopherol In this instance the thesis that a deficiency of vitamin E may exist in the tissues even though the blood contains a measurable tocopherol is untenable The rabbit is more sensitive than most animals to a dietary deficiency of vitamin E On the basis of these measurements, it appears that low or even zero serum tocopherol levels are not indicative of avitaminosis E On the other hand, Dr Gyorgy has shown that a serum tocopherol level that is compatible with reproduction of the rat does not suffice to protect against *certain hemolyzing agents* Clearly, as is the case with every vitamin one should not speak of adequate or inadequate tocopherol blood levels without saying adequate or inadequate for what

Dr Pappenheimer Have you any explanation for the failure of rabbits to develop dystrophy on diet 11?

Dr Mackenzie I believe that Dr Mason feels that the difficulty now encountered in producing muscle lesions in suckling rats may be related to a change in the processing of lard I might add that when the rabbits with essentially zero blood tocopherol levels were transferred to your ferric chloride treated diet they developed acute dystrophy in 3 or 4 days

Dr Ling Dr Gyorgy mentioned that peroxide in the right case may serve as a bioassay for vitamin E level in the blood I don t think it is feasible, for

there are several reducing agents present in the blood particularly in the blood cells such as glutathione and ascorbic acid as Dr Gyorgy mentioned in his lecture Therefore unless one determines simultaneously all of the reducing agents present in the blood I don t think one can do bioassay on vitamin E level in the blood Secondly we know that there are many ways that one can produce necrosis of the liver such as injections of carbon tetrachloride or even India ink Dr Himsworth s work shows that necrosis of the liver regardless of its causative agent is due to the swelling of the liver cells thereby causing anoxia in certain parts of the liver lobules I think the dietary necrosis Dr Gyorgy mentioned may be due to a different mechanism than anoxia Dr Gyorgy did not specify what kind of diet he used One can produce liver necrosis with various types of diet for example diet containing a large amount of fat I would like Dr Gyorgy to clarify whether vitamin E can always prevent necrosis of the liver or just what type of necrosis of the liver it can prevent

DR GYORGY I would like to clear up a misunderstanding Bioassay in vitamin research means analytical determination of a vitamin with the help of living test organisms (animals microorganisms) The bioassay of vitamin E based on the hemolysis test includes the use of animals With regards to the types of necrosis the fat content of the diet is not the determining factor Low protein *high* fat diet is used for the production of hepatic cirrhosis In Himsworth s yeast diet which I also employed in my studies on dietary necrosis the fat content is very low 5 per cent Vitamin E prevents this dietary necrosis but it is no reason to believe that in this effect dietary fat is involved

DR LING Dr Himsworth in his lecture did not distinguish any difference in the liver necrosis produced by different types of diet But he did mention that in one of his experiments addition of cystine produced liver injury because cystine promoted growth of the rat and thereby produced a relative deficiency in lipotropic factor (H P Himsworth *The Liver and Its Diseases* pp 67 69 Harvard University Press 1947)

DR GYORGY Well I think I ll have to defer this discussion

DR MASON May I add just one comment I didn t want to leave the impression that determination of plasma tocopherol levels has no value and I think Dr Gyorgy understands that Certainly tocopherol plasma levels when they are low have real significance When they are high they may have significance When they are within the normal range they may not tell us very much of the story but perhaps give us an assumption which is reasonably adequate But on top of that the tocopherol response curve whether values are low or normal may give us further insight into the mechanisms of storage in the body

Dr Hickman May I comment on a slide of Dr Mason s and address a question to him? I have arranged to have the slide projected I want to attempt an explanation of this chart which displays the tocopherol levels in the blood before and after parturition The theory that intrigues me is the fact that the blood tocopherol of a non nursing mother goes down faster than that of a nursing mother To me, this happening carries a definite message We have always wondered—and it seems a very wise provision of nature that the maternal blood level of tocopherol should rise in order to push the vitamin E across the placenta to the child—we have always wondered what was the actual mechanism for the rise Let us postulate a mother so carefully groomed by the doctor that she puts on no weight during pregnancy Surely the fetus will then grow chiefly at the expense of the host Now the host s—and indeed anybody s—muscular tissue is known to contain a much higher concentration of tocopherol than the blood, therefore, as the tissue is used up building the fetus the tocopherols will be liberated but have nowhere to go except into the blood stream where the concentration will rise And in the same way, if during nursing the fat depots of the mother are consumed to provide fat for the milk, tocopherol will also be released This will not happen in the case of the non nursing mother Of course in the normal situation, the mother increases in weight during pregnancy but we can still apply the hypothesis as we make two assumptions, the first being that my argument holds in part anyway and the second that the fetus is nourished primarily from tissues of the host, which are in turn replenished from the host s digestive tract The *old* tissue, at the expense of which the child is growing is rich in tocopherol, through years of endowment, while the new tissue which is being replaced from food is at first low in tocopherols The net, overall transaction is to push tocopherol into the blood stream

Forgive me Dr Mason, but I enjoy constructing hypotheses, particularly when someone else has done all the work!

Dr Schwarz In Dr Gyorgy s results I found that the most striking feature is that the germ free animals grow whereas the controls do not The germ free animals grow on a diet which apparently is quite limited in sulfur amino acids In normal animals cystine which is known to be active as a protective factor against liver necrosis will promote growth when added to these diets (A Hock and H Fink, *Ztschr physiol Chem* 278, 136 (1943)) The question arises whether it is possible to explain Dr Gyorgy s findings on the assumption that the intestinal flora competes with the host for sulfur containing animo acids I would furthermore, think that it would be important to know about food consumption in these experiments We have observed that the development of dietary necrotic liver degeneration is strongly inhibited when rats on yeast diets are kept on a very low food intake (unpublished data)

DR GYORGY Part of my talk today was only a condensed version of a talk I gave last Monday in New York at the American Chemical Society where I discussed the possible explanation for the findings in the germ free animals as well as of other similar findings According to this explanation the intestinal flora may either reduce the amount of essential dietary constituents available to the host organisms or it produces metabolites toxic to the liver In germ free animals or under the influence of antibiotics this injurious role of the intestinal flora is eliminated or delayed

DR SCHWARZ In addition, I might mention that we have recently discovered another dietary factor (factor 3) involved in the prevention of necrotic liver degeneration (*Proc Soc Exp Biol and Med* 78, 853 (1951)) A competition between host and intestinal flora for factor 3" might also be involved, offering another possible explanation for the differences observed by Dr Gyorgy between germ free animals and controls

PATHOLOGICAL EFFECTS RESULTING FROM DEFICIENCIES OF INORGANIC ELEMENTS

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SOME YEARS AGO the following statement (1) appeared in the *Journal of Biological Chemistry* That the sodium, potassium calcium magnesium phosphate, chlorine and sulfate ions play an essential role in living organisms is a fact generally accepted This was written by Dr McCollum in 1915 and exemplifies the interest he has continually displayed in the importance of the inorganic elements in nutrition

At the present time there are 17 metallic and nonmetallic elements which are essential for the mammalian organism In addition an eighteenth, fluorine, is also probably indispensable One can group these essential elements in an old fashioned periodic table as shown in Fig 1 In this table the indispensable elements are printed in bold face type, those indispensable for plants appear in italics, while those which are ubiquitous are in normal type face and those not found in the organism are in small face It is obvious that there is not enough space in this paper to discuss all of the essential elements at length I have chosen, therefore to take up only three all of which have been studied in Dr McCollum s laboratory and with which I have had some first hand knowledge

The first of these is potassium an element which because of its intracellular position is of great importance to the tissues and which today is assuming more and more importance in clinical medicine The effects of potassium ions on the heart were clearly shown towards the end of the last century by Sidney Ringer (2) In 1941 Dr Orent Keiles and Dr McCollum (3) were able to prepare a diet which contained only 01 per cent potassium We were fortunate to be able to study rats which had been placed on this diet for varying periods of time It was extremely interesting to find that

very early in the deficiency damage to the heart muscle fibers could be demonstrated morphologically (4) After the first week certain fibers lost their striations and assumed a hyaline appearance This change was accompanied by cellular infiltration so that very soon the picture resembled that of a diffuse myocarditis Such animals did not ordinarily die but as time went on, up to over 200 days on the deficient diet, necrotic myocardial fibers were replaced by scar tissue

GROUP	I	II	III	IV	V	VI	VII	VIII
PERIOD								
1	H							
	Li	Be	B	C	N	O	F	
2	Na	Mg	Al	Si	P	S	Cl	
3	K	Ca	Sc	Ti	V	Cr	Mn	Fe Co Ni
	Cu	Zn	Ga	Ge	As	Se	Br	
4	Rb	Sr	Y	Zr	Cb	Mo		
	Ag	Cd	In	Sn	Sb	Te	I	
5	Cs	Ba						

FIG 1 Abridged periodic table of the elements

One of the most interesting and unexplained features of this picture is why some myocardial fibers are affected during the first week and why 30 odd weeks or more must elapse before other are damaged

In the rats placed on the potassium low regimen lesions of skeletal muscle did not appear, even when the animals were subjected to daily exercise (5) The only other tissue found to be involved was the kidney Neutral fat appeared in the renal tubular epithelial cells before the first week was over and such cells became necrotic and were sloughed off into the lumen of the tubule Some of them appeared to calcify At the end of several weeks one encountered prominent dilated tubules and calcified casts in the collecting structures in the pyramids

At the time these observations were being carried out on potassium deficiency in rats somewhat similar studies were going on in associa

tion with Dr M M Wintrobe in thiamin deficient swine Because identical cardiac lesions were encountered in this spe was only natural to put the two deficiencies, potassium and thia together in order really to wreck the myocardium Much t surprise no lesions at all appeared (7) *What did happen, ho* *was that necroses of the skeletal muscle fibers presented them* in animals on the doubly deficient diet

The studies on the potassium deficient diet in rats led us to ex another relationship, that of the possible substitution of rubidiu *ceseum in the potassium low ration Accordingly these two ele* were added to the diet in place of potassium (8) It was foun rubidium and to a lesser extent ceseum prevented the appearan the characteristic cardiac and renal lesions of potassium deficien

Shortly after the demonstration of morphological chang potassium deficiency in the experimental animal the importan this ion for the integrity of human tissues became apparent H kalemia became something to take into consideration in pa being treated for diabetic coma (9) So too, the importance o loss of this element from severe vomiting or diarrhea became app (10) Renal disease may also alter serum potassium levels (Today in the clinical chemical laboratory numerous serum potas levels are doubtless being determined

The second element which I should like to discuss is zinc, w of course, is a typical trace element In 1934 Todd, Elvehjem Hart (12) prepared a diet extremely low in zinc content In Drs Harry Day and McCollum (13) concocted a ration furnis only 1 to 4 micrograms of this element per day In association Drs Day and McCollum we (14) were able to study the tissue such animals Two areas were found to be affected—the skin of all places, the esophagus Fairly early in the deficiency the over the dorsum began to fall out and the epithelium became crus Microscopic examination revealed a thickened acanthotic epith layer covered with a crust of partially keratinized cells The appa cause of the loss of hair was atrophy of the hair follicles In cont the sebaceous glands were increased in size in comparison with normal This dermatological picture is quite unique and spec

In our experience similar changes have not been produced by other nutritional deficiencies in rats

In the esophagus, alterations were encountered which have not been described as a result of any other nutritional deficiency Here one found an increase in thickness of the epithelial lining cells together with the presence of large partially keratinized cells on the surface The basal cells were more numerous and closely packed The change must be interpreted as being due to a retardation in normal keratinization

With the exception of the cornea which showed vascularization in two of the seven zinc deficient animals which were studied all of the other tissues revealed nothing save nonspecific changes which were related to general inanition

The third element which I should like to discuss is iodine It is particularly appropriate to take up iodine because a most far reaching and important observation dealing with the metabolism of this element was made in Dr McCollum s laboratory by Dr and Mrs Mackenzie (15)

Iodine deficiency is usually said to be the cause of goiter At this point it is well to stop and call attention to and plead for care in the use of the term, goiter Swelling of the neck may be of two types The first, colloid goiter, is due to a filling up of the thyroid follicles with relatively iodine poor colloid The second type, hyperplastic goiter, is due to a proliferation of epithelial elements and an increased vascularity of the tissue This type of gland is poor in its colloid content

In experimental animals iodine deficiency, which has been studied by a number of investigators leads to the second type of goiter, *the hyperplastic type This apparently results from a deficiency in* production of the active principle of the thyroid gland which results in an increased production of thyroid stimulating hormone by the hypophysis The other type of goiter, the colloid form, is usually ascribed to iodine deficiency also Such a concept is based on the occurrence of so called endemic goiter which is associated with areas whose soil and water are low in iodine content A great deal of confusion has arisen because those who have made surveys of the

incidence of goiter in such localities have used the term indiscriminately, not determining the precise nature of the enlargement of the neck, i e whether it was due to colloid deposition in or hyperplasia of the gland

Colloid goiter has never been produced in the laboratory, the only approach to anything resembling this change may be found in animals to which thyroid extract has been administered It is quite possible that if iodine deficiency is related to colloid goiter in the human that other factors may also be acting as adjuvants It is well known that high concentrations of calcium produce goiter So too, cabbage contains a goiterogenic substance Greenwald (16) has discussed the problem of iodine and colloid goiter at some length and has concluded that a great deal still needs to be learned concerning the relationship of iodine deficiency to colloid goiter

There is another mystifying relationship of iodine and the thyroid gland, the response of the hyperplastic and extremely physiologically active tissue to the therapeutic administration of iodine As is well known, when iodine is given to a patient with Grave's disease the basal metabolic rate is lowered and other effects of overstimulation of the tissues by active principle are reduced In addition and undoubtedly related to the decrease in output of thyroid hormone conspicuous morphological changes appear in the gland as Rienhoff first showed a number of years ago (17) The follicles fill up with colloid and the extremely hyperplastic cells appear morphologically less active We cannot go into the possible causes of this change The subject has recently been reviewed by Astwood (18) The conclusion reached is that we just don t know The relationship is, of course, further complicated by the fact that iodine has only a transitory effect, not longer than a month

Lastly I should like to point out the far reaching effects which the Mackenzie s (15) demonstration of the antithyroid effects of certain chemical compounds has had on the pathological laboratory In the first place the introduction of these compounds has reduced the number of specimens reaching the laboratory, and secondly therapy with such drugs completely changes the morphology of the tissues we do receive Some years ago a favorite pastime was to

try to gauge the basal metabolic rate by the degree of thyroid hyper plasia After Dr and Mrs Mackenzie demonstrated that certain chemical compounds produced extreme hyperplasia of glands which were hypofunctioning it because only too apparent that the gland morphologically might be hyperplastic yet the patient could be hypothyroid, euthyroid or hyperthyroid

In closing I should like to read something which Dr S Burt Wolbach (19) wrote a number of years ago, A general pathologist who studies life chiefly from the morphological alterations may well be apalled by the wasted opportunities represented by animals con signed to incinerators at the completion of carefully conducted experiments in nutritional fields Dr McCollum is very definitely one investigator to whom this statement would not apply

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17 Rienhoff W F *Bull Johns Hopkins Hosp* 37 285 (1925)
18 Astwood E B *Ann N Y Acad Sci* 50 419 (1949)
19 Wolbach S B *Nutr Rev* 3 193 (1945)

DISCUSSION

DR HICKMAN In the case of vitamin deficiency if you give extra vitamins you cure presumably, the condition And then after that you can give very large quantities of vitamin It s virtually inactive up to a thousand times or

more the required dose With these trace minerals, after you have cured the deficiency, what s the size of the dose that becomes toxic? Do they become toxic in large quantities?

Dr Follis I don t think I can answer that question Perhaps Dr McCollum can I think certainly that you can kill the organism if you saturate it with some elements I m thinking now of calcium or phosphorus You stop up the route by which they re excreted and probably kill the animal in that way Zinc of course is toxic in large quantities, you can have zinc poisoning

Dr Kramer Surely Dr Hickman knows that potassium may be very toxic

TRACE ELEMENTS IN HUMAN NUTRITION

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THE WELL KNOWN contributions of Professor McCollum conce the nutritive significance for animals of trace inorganic subst such as manganese, aluminum, zinc, fluorine and iodine (i e st on goitrogens) are responsible for the inclusion of the title of paper in a conference which bears testimony to Doctor McColl influence upon the science of nutrition It is a privilege to be all to participate in this symposium

This discussion is to be regarded as an editorial treatment c unsatisfactorily developed field It will attempt to indicate are sound knowledge, of uncertainty of knowledge, of gross ignor and of popular faddishness The attached bibliography is inte to be illustrative only, and is recognized as incomplete

This assessment of the present state of our knowledge of elements and human nutrition has led this reviewer to conc that (1) there exists a body of sound knowledge regarding a lir number of elements which requires practical development, (2) is a void of information regarding many substances, (3) and nu ous weird claims have been made upon the basis of our ignoran

As a basis for discussion, certain concepts are important

(1) The diets of the human even the more restricted diets not comparable in their limitations to those rigorously purified which are employed in laboratory experiments for the productic animals of deficiencies of trace elements Accordingly, the likeli of the occurrence of a dietary deficiency of trace elements in is greatly reduced In many areas of the world this likelihood dietary deficiency of trace elements in the human is further i mized by the inclusion in the diet of a variety of animal produc which a considerable concentration of a scarce element has alr

taken place As noted by L A Maynard (1950), this relative constancy of composition of foodstuffs of animal origin is important in assuring a reasonable supply of a mineral in the human dietary despite a relative paucity of the substance in the soil of a region Hence, the presence of an element in human tissues cannot be accepted as evidence that a deficiency of the element is likely to occur, even though the element has been demonstrated to be essential for experimental animals under rigid laboratory conditions

(2) In studies designed to detect human dietary deficiencies it is essential to appraise soundly the methods used and the interpretations which can validly be made of a given set of data

(a) In epidemiologic investigations totally unrelated phenomena may be correlated Correlation of such factors does not signify causation

(b) The technic of metabolic balance is a most valuable tool for the study of requirements, but retention of an element does not *signify that the element is a dietary essential* *Unfortunately,* the wisdom of the body" does not permit it to retain only essential substances Furthermore, essential substances may often be retained *by the body in excessive amounts*

(c) Therapeutic testing or the effect of dosing with an element may be misleading, for responses may be either nonspecific or toxic in character For example, arsenite may produce a reticulocyte response in normal subjects or in patients with *pernicious anemia,* it may depress the leukocytes in patients with myelogenous leukemia, and it may lower the erythrocyte count in persons with polycythemia (Minot and Castle, 1935) Yet these effects of arsenic are not likely to be due to replacement of a deficiency!

(3) Finally, as Doctors McCollum, Day, and Orent-Keiles (1939) have noted, There are two aspects of the problem of trace elements in relation to health,—one is that of the nutritional need of the animal organism and the other that which is manifested in industrial hygiene, agriculture, and in foods, namely, the possibility of intoxication due to any one of these minerals" With the inorganic nutrients more than with some other factors this zone of excess is real and of practical importance

IODINE

Numerous excellent recent treatments of the subject of iodine metabolism and the thyroid (Means, 1948, Curtis and Fertman, 1951, Salter, 1950, Leblond, 1948, Rawson and Money, 1949, Keating and Albert, 1949, Seidlin, 1949, Werner, Quimby, and Schmidt, 1948, Astwood, 1949, Greer, 1950, Pitt Rivers, 1950, Barker, 1951) render trite and unnecessary a detailed summary of our present knowledge of the metabolism of iodine Suffice it to indicate that iodine is an essential nutrient for animals and man (Sebrell, 1949) The major known role of iodine in the human organism is in the formation of thyroid hormone The magnitude of the requirement for iodine was estimated by Curtis and Fertman (1951) to be of the order of one to two mcg per kg of body weight per day, and slightly higher by the Food and Nutrition Board of the National Research Council (1948)

In the United States there has been a considerable tendency to regard the problem of goiter control as solved by the use of iodized salt (Kimball, 1939, 1946) This widely accepted viewpoint is illustrated by the statement contained in the 1948 revision of the Recommended Daily Allowances The requirement for iodine is small, probably about 0 002 to 0 004 mg daily for each kg of body weight, or a total of 0 15 to 0 30 mg daily for the adult This need is met by the regular use of iodized salt its use is especially important in *adolescence* and *pregnancy* The relative infrequency with which endemic goiter is now met in the United States has encouraged the general acceptance of this simplified viewpoint

The observed decrease in endemic goiter in this country has not been a phenomenon shared by the majority of the world population In the summer of 1951, the Chief of the Nutrition Section of the World Health Organization Doctor F W Clements stated to me that he regarded endemic goiter as probably the world s most prevalent deficiency disease It may indeed be a revelation to other workers in this country, as it was to me, to see the extent and severity of goiter in adults and in quite young children which one encounters

in many areas of the world. The accompanying photographs were provided by the Institute of Nutrition for Central America and Panama in Guatemala City and illustrate this point. I have observed recently similar examples in Austria and in Croatia and Serbia in Yugoslavia. In these latter regions one occasionally encounters cretinism.



FIG 1 Endemic goiter in a group of women in a goiterous region of Guatemala (Photograph through the courtesy of the Institute of Nutrition for Central America and Panama, Doctor Nevin Scrimshaw, Director)

A review of the reports of medical workers and a description of nutritional survey findings from large areas of the world indicate that such examples as these are widespread indeed.

What control measures will be efficacious in these regions? Why have not such measures been instituted? Sound categorical answers cannot be given to these questions The following are some of the considerations which arise as one attempts to answer them:

Are these goiters due to iodine deficiency? It has been concluded by at least one reviewer (Greenwald, 1946) in recent years that " endemic goiter is not due to a lack of iodine." The present writer does not believe that the arguments which have been advanced are

valid support for such a conclusion but it is certain that all of the factors which influence the appearance of goiter in a region have not been defined A major point in Greenwald s argument has been



Fig 2 A large go ter in a 52 year ld re dent of Panama (Photograph through the courtesy of the Inst tute of Nutr t on for Central Amer a and Panama Doctor Nev n Scr mshaw D rector)

that there exists a lack of correlation between iodine supply and goiter Let us examine this point further

The epidemiologic association of goiter with certain types of environments particularly the older mountainous regions has been long recognized McClendon and Williams (1923) indicated broad

inverse relationships between goiter rate in recruits for the first World War with the iodine content of representative river waters of the United States This is indicated by the familiar map which accompanies their publication It is sometimes overlooked that these workers were *not* attempting to determine the amount of iodine supplied by the water but, instead, merely employed the iodine content of river water as an indicator of the probable available iodine in the region The existence of any correlation is, then, the more significant

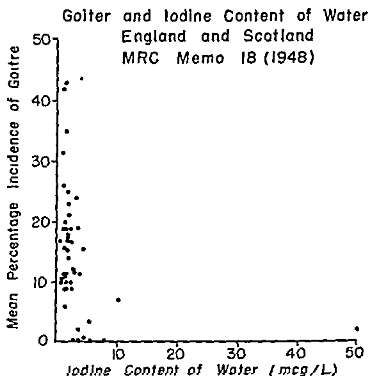
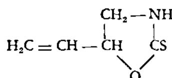


FIG 3

It is of value to examine recently accumulated data published in 1948 on the relationship between the iodine content of drinking waters in various regions of England and Scotland and the percentage incidence of "visible thyroid glands" or of "goiter" (Murray *et al*, 1948) These data (Fig 3) again indicate that there is a greatly reduced incidence of goiter in regions where the iodine content of the water is high

The absence of a fixed relationship between the iodine content of drinking waters and the incidence of goiter is to be expected when one reflects that in most areas the major supply of iodine must come from foods and, hence, the nature of the food supply determines the iodine intake

Further, a growing mass of data indicates that the nature of the food supply may determine the amount of iodine required for the prevention of goiter The most acceptable evidence for this view stems from the observation of Chesney, Clawson, and Webster (1928) of the occurrence of goiter in their colony of rabbits Two years later, Webster and Chesney (1930) concluded that the feeding of cabbage was responsible for the epizootic of goiter in their rabbit colony Interest in the goitrogenic agents was greatly stimulated by the observation of MacKenzie, MacKenzie and McCollum (1941) that the prolonged administration of sulfaguanidine to rats would produce goiter In this instance the administration of iodide does not appear to be effective in the prevention of formation of the goiter (MacKenzie, 1947) It would be out of place to attempt to review the tremendous developments in the anti thyroid agents for therapeutic purposes It is pertinent, however, to emphasize that Astwood, Greer, and Ettlinger (1949) have isolated the goitrogen 1 5 vinyl 2 thiooxazolidone from the rutabaga and other members of the genus *Brassica* This goitrogen is stated to be equal in potency to thiouracil in man Added iodine does not abolish the goitrogenic effect of feeding with *Brassica* seed (Greer, 1950) and, hence, presumably does not counteract this particular goitrogen On the other hand, the goitrogenic effect of thiocyanate is observable only when iodine intake is quite low



1 5 vinyl 2 thiooxazolidone

(Astwood Greer and Ettlinger *J Biol Chem* 181 121 (1949))

The extension of studies of the naturally occurring goitrogens and their effect on iodine requirements may assist us to understand the lack of fixed iodine requirements under all conditions As pointed out by Greer (1950), the relationship of other nutritional factors, such as high protein diet, high fat diet, high carbohydrate diet, vitamin deficiencies, etc must be reinvestigated, employing newer technics based upon present-day knowledge before their role in goiter production can be assessed

Despite the incompleteness of our knowledge of the factors which determine the requirement of iodine and the development of goiter, it is germane to keep clearly in mind the effectiveness of iodine administration in the prophylaxis and treatment of endemic goiter The classical demonstration by Marine and Kimball (1920) of the prophylactic effect of the administration of 2 g of sodium iodide in 0 2 g daily doses for ten consecutive school days each spring and autumn to pupils in the Akron public schools is based on 2,190 treated children and 2,305 untreated controls These data are summarized in Tables 1 and 2 and can leave no doubt as to the effectiveness of the administration of iodine in the prevention and treatment of goiter

TABLE 1

PERCENT OF CHILDREN SHOWING *Decrease* IN SIZE OF THYROID WITH AND WITHOUT TREATMENT

(Sodium iodide 0 2 g per day for 10 days by mouth at 6 month intervals)

Time under observation	Original thyroid state			
	Slightly enlarged		Moderately enlarged	
	No treatment	Iodine	No treatment	Iodine
mo				
6	3 7	29 5	0 0	18 2
12	19 9	54 5	3 8	76 2
18	13 1	46 7	26 3	72 0
24	14 3	61 6	20 0	92 3
30	10 5	71 5	33 3	97 4

(Marine and Kimball, *Arch Int Med* 25, 661 (1920)

In an effort to apply this knowledge to the prevention of goiter in population groups, several vehicles for carrying iodine have been suggested Among these may be mentioned confectioneries, water,

milk, and salt Despite the enthusiastic endorsement by many official and nonofficial agencies in the United States of iodized salt as the practical solution of the goiter problem and the enthusiastic reports of its effectiveness, reports of failure with this agent are too numerous to ignore For example, Hercus and Purves (1936) stated that ' The regular use of iodized salt of the present New Zealand standard for domestic purposes is not an efficient protection against goiter "

TABLE 2

PERCENT OF CHILDREN SHOWING *Increase* IN SIZE OF THYROID WITH AND WITHOUT TREATMENT

(Sodium iodide 0 2 gm per day for 10 days by mouth at 6 months intervals)

Time under observation	Original thyroid state					
	Normal		Slightly enlarged		Moderately enlarged	
mo	No treatment	Iodine	No treatment	Iodine	No treatment	Iodine
6	50 0	5 6	26 9	1 3	30 4	0 0
12	24 5	0 3	9 8	0 0	30 8	0 0
18	34 8	0 0	12 3	0 7	15 8	0 0
24	23 3	0 0	6 0	0 5	20 0	0 0
30	24 9	0 0	15 8	0 0	0 0	0 0

(Marine and Kimball, *Arch Int Med* 25, 661 (1920)

Where iodized salt has been employed, there has been great variation in the level of iodinization In the United States the level of iodinization has been established at one part of potassium iodide or suitable equivalent for each 10,000 parts of salt, representing 75 to 80 parts iodine for each million parts of salt Several groups (Editorial, 1947) have endorsed the definition of iodized salt as salt carrying not less than 75 and not more than 150 parts of iodine as iodide for each million parts of salt On the other hand, in much of the rest of the world the iodinization of salt has been at a level of one part of iodine in some 100,000 to 250,00 parts of salt

Osmond and Clements (1948) in a careful study of the problem of iodine prophylaxis of endemic goiter in Canberra have shown that salt consumption, especially by children, is much more variable than most of us suspect From consumption studies in 175 house holds, the minimal daily consumption of table salt per adult male

unit was 0 96 g, the maximum consumption 20 8 g, and the mean daily consumption 5 47 g Observations of the children showed that many did not use table salt The number of children not using salt decreased yearly from 1 to 9 years of age, but after 9 years some 10 per cent still used no salt (see Table 3) Obviously, iodized salt at the table alone at any level of iodinization cannot serve as an effective prophylaxis against goiter for 30 to 35 per cent of the children at 3 to 4 years of age or 10 per cent of the children after 9 years of age

TABLE 3

PERCENT OF 1,241 CHILDREN WHO DID NOT USE SALT AT THE TABLE

(Osmond and Clements 1948)

Age in years	Per cent
1	75 0
2	41 0
3	35 0
4	32 6
5	15 7
6	24 0
7	15 5
8	10 2
9	10 5
10	13 6
11	7 9
12	10 9

Again, these studies revealed that the average daily intake of table salt by children who used salt regularly is approximately 0 8 of a gram It was calculated that this quantity of salt as iodized would provide (a) 8 0 mcg of iodine at a concentration of one part in 100,000, (b) 80 mcg of iodine at a concentration of one part in 10,000, (c) 160 mcg of iodine at a concentration of one part in 5,000

Such a simple but direct analysis serves to convince this reviewer that iodized salt for table use alone cannot solve the goiter problem It would be less effective at the low levels of iodinization in use in some regions

Clements has pointed out that the stability and retention of iodine in salt during cooking requires attention

A practical problem in the use of iodized salt for many areas of the world has to do with the stability of iodine in crude salt It is probable that the largest portion of the world s population consumes crude salt instead of a refined product The stabilization of iodine in crude salt so that it is economically feasible for these areas remains to be developed

Finally, it must be recognized that a fear of precipitation of toxic symptoms by the administration of iodine (Jod Basedow s disease) makes some oppose iodinization at higher levels This is especially true in Europe Demonstration studies are needed to decide the issue convincingly in these regions

These considerations which I have raised in relation to iodized salt are not intended to discourage the use of this vehicle where it is practical They are intended to remind us that it is an over simplification of fact to assume that the world s goiter problem will be solved by mere education in the use of iodized salt We must have studies along the lines indicated if we are to solve this, the world s most prevalent deficiency disease

Fluorine

Another halogen of interest in a discussion of trace elements in man is fluorine Knowledge of the role of fluoride in the human has accumulated primarily as a result of epidemiologic studies The fundamental investigations relating fluoride ingestion to mottled enamel and to dental caries are well summarized in the two monographs of the American Association for the Advancement of Science (Moulton, 1942, 1946)

The geographic distribution of mottled enamel suggested its association with water supplies of high fluoride content The production of analogous dental conditions in experimental animals fed on high levels of fluoride and the prevention of mottled enamel by change in water supply from high to low fluoride sources provided adequate evidence of the relationship

Epidemiologic studies have served as the basis for the concept of a relationship between fluorine in drinking water and a low dental

caries incidence (Moulton, 1946) Such investigations have led to wide acceptance of the conclusions that mottling of the enamel is rare and mild in individuals who drink water containing 1 p p m * of fluoride Furthermore, the lowest dental caries rates are encountered in areas where the water supply has a naturally occurring fluoride content of about 1 p p m These observations are strengthened by the observed reduction of dental caries by approximately 40 per cent after topical application of fluoride Accordingly, there has been developed the process of fluoridation of city water supplies as effective cheap methods for the reduction of dental caries (Council on Dental Health, 1951)

A number of test situations have been devised to determine whether the addition of fluorine to the water supply of fluoride free areas at the optimal level of 1 p p m would decrease the dental caries rate among children in the water supply area One such study is that in the cities of Newburgh and Kingston in New York State On May 2, 1945, sodium fluoride additions to the water supply of Newburgh were initiated and the water supply of the control city of Kingston remained fluoride free Preliminary data on the results of the first three years of water fluoridation in these areas have appeared (Ast, Finn, and McCaffrey, 1950, Schlesinger, Overton and Chase, 1950), and they do indicate that a consistent downward trend in the DMF (decayed, missing, filled) rate for permanent teeth has occurred in Newburgh, the treated city Similar encouraging results have been noted in studies elsewhere (Dean *et al*, 1950) To date, all of the investigators emphasize the necessity of prolonging the periods of observation to obtain conclusive proof of the efficacy of this prophylactic measure In fact, Ast and coworkers, in speaking of the Newburgh Kingston study, conclude that These data are preliminary and it will be necessary to continue collecting data for the proposed duration of the study, that is, through 1954 to 1956, to obtain additional information concerning the caries prophylactic value of fluorine

How close to the level of chronic toxicity is the provision of water

* p p m = parts per million

containing approximately 1 p p m of fluoride? Epidemiologic experience demonstrates that this is the border zone, submarginal for the production of dental fluorosis The index of dental fluorosis

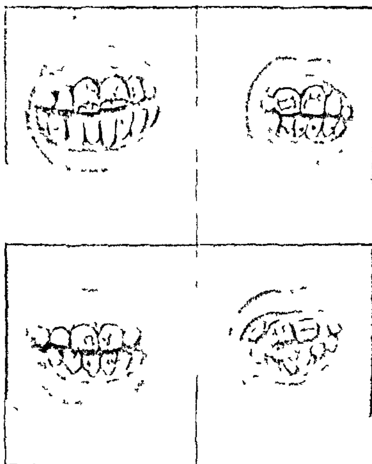


FIG 4 Mottled enamel in children in an area of endemic fluorosis in Madras Presidency (After Shortt *et al* 1937)

(an expression of both frequency and severity of mottled enamel) begins to rise somewhere between a fluoride content of 1 to 2 p p m (Dean, in Moulton, 1942)

Investigations of the excretion and distribution of fluorine (McClure, in Moulton, 1946) reveal that teeth and bone are more sensitive to fluorine and retain it to a greater extent than do other

tissues of the body. Studies of fluorine balance indicate that, in adults, 90 per cent or more of the ingested fluoride is excreted if the quantities ingested do not exceed 3 to 4 mg daily. This is equivalent to the drinking of 3 or 4 liters of water containing one part per million of fluorine or of 2 liters of water per day at the level of

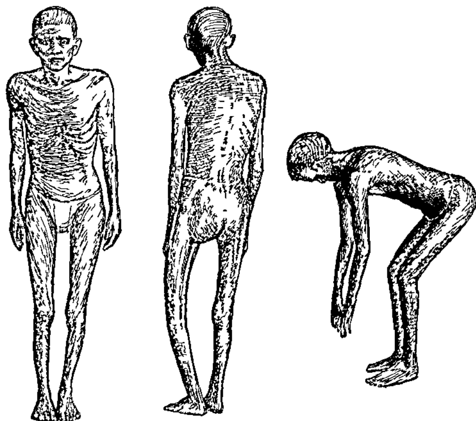


FIG 5 Characteristic postures in endemic fluorosis Note cachexia flat chest, stiff intercostal muscles, rigidity of the back and inability to squat (After Shortt *et al*, 1937)

2 p p m The daily ingestion of 6 mg of fluorine did result in retention of a few milligrams of fluoride, the greatest retention resulting from ingestion of the substance in drinking water (Machle, Scott, and Largent, 1942, 1943)

Are there toxic effects of fluoride ingestion other than mottled enamel? Møller and Gudjonsson (1932) described osteosclerosis with calcification of ligaments and muscle attachments, gastroin-

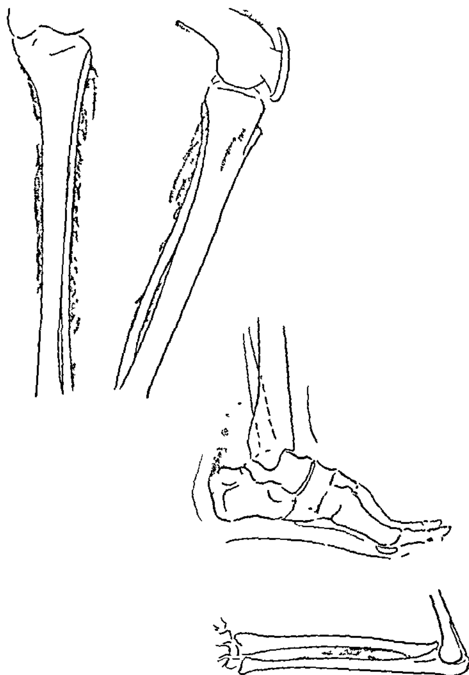


FIG 6 Drawings of X rays showing osteophytic outgrowths of bone (dotted areas) (After Shortt *et al*, 1937) Note rose thorn appearance of the long bones and the calcification of the Achilles tendon

testinal symptoms, and anemia in 39 of 78 Cryolite workers Bone changes are part of the picture of fluorosis in animals Similar findings have been reported from South India (Shortt, Pandit, and Raghavachari (1937), Raghavachari and Venkataramanan (1940), and Pandit *et al* (1940)) These changes are illustrated in Fig 5 and 6 This endemic fluorosis occurred in populations using drinking water with a fluoride content of from 1 to 6 p p m In these studies, about three-fourths of the children showed mottled enamel even at fluoride concentrations of one part per million The severe bone manifestations of chronic fluoride intoxication appeared only in persons who had resided continuously in the area from childhood and exposed themselves to the high fluoride consumption for 15 to 25 years About three fourths of such persons showed these evidences of bone changes

Actual quantities of fluoride ingested were estimated to vary between approximately 5 and 24 mg per person per day Severest involvement of bone ligaments and joints was associated with water supplies containing the highest fluoride content of about 6 p p m Finally, there existed no fixed relationship between the fluoride consumption and the degree of fluorosis Economic and nutritional surveys pointed to the possibility of a non fluoride nutritional factor determining the severity of the fluorosis The authors suggested that a pronounced deficiency of ascorbic acid might be one such factor This latter suggestion was tested by a study of the toxicity of fluorine in monkeys with and without ascorbic acid (Pandit and Rao, 1940) It was found that the ascorbic acid deficient animals showed greater absorption of fluorine, greater excretion, and more marked radiologic changes It is possible, of course, that this observation is merely related to the changes in ossification which occur in scorbutic animals

Studies of 117 individuals in the United States consuming water containing 1 to 3 parts per million of fluorine failed to reveal evidences of bone changes (Hodges *et al*, 1941) If the absence of some nutritional factor does indeed influence the severity of bone lesions due to fluorosis, it may be that the nutritional level in this country is favorable for the prevention of such alterations

Information available at the present time indicates that the chief

source of available fluoride for the human is water Although the data are not extensive they indicate that the fluorine content of usual foods would make but a slight contribution to the daily intake of this element Furthermore it does not appear that plants tend to absorb large quantities of fluorine from soils high in content of this element

A number of organizations including the American Public Health Association American Dental Association and State and Territorial Health Officers concerned with professional health work have within the past year passed resolutions supporting fluoridation of water supplies as a public health measure It is this reviewer s opinion that unqualified recommendations of this procedure as a safe and effective method for reducing the prevalence of dental caries could be more soundly given had the present pressure in favor of fluoridation not developed until after (1) we had accumulated additional evidence in the existing studies of the effectiveness of fluoridation procedures (2) further investigations of the accumulation of fluorides at low levels of fluoride ingestion had been made especially in children (3) such a time as to permit observations on possible skeletal changes after 10 to 15 years of fluoridation experience and until additional investigations of the possible occurrence of osteosclerosis were made in areas of high fluoride consumption in the United States This represents additional information which must be accu mulated before we can give unreserved support to fluoridation as a public health measure

Is fluorine an essential trace element for man? Despite the exten sive studies of the biological effects of fluorine in animals and in man we cannot as yet state that the element is an essential nutrient In 1933 Doctors Sharpless and McCollum succeeded in rearing rats on a diet very low in fluorine but not quite free Evans and Phillips (1939) reared 5 generations of rats on mineralized milk and con cluded thereby that 50 mcg of fluorine per kilogram of body weight per day suffices for growth reproduction and well being of the rat

Cobalt

There are three well established observations concerning the nutritional importance of cobalt which bear directly upon considerations of this metal as a trace element for man (1) A lack of cobalt in the diet of ruminants produces a deficiency syndrome characterized by anemia and other symptoms So far as I have been able to find, there are no reports of the production of cobalt deficiency in nonruminating animals (2) Administration, orally or parenterally, of excessive quantities of cobalt to experimental animals produces a true polycythemia (3) Vitamin B_{12}, a hemopoietic factor, contains cobalt (Smith, 1948, Rickes *et al*, 1948) and this vitamin is required by or exerts a physiologic effect in most species of animals under one or another condition

The most recent reports are consistent with the hypothesis that cobalt is required by the ruminant in order that the synthesis of vitamin B_{12} can be accomplished by micro organisms within the gastrointestinal tract of these animals (Smith, Koch, and Turk, 1951) It has been demonstrated that injected vitamin B_{12} will permit hemopoiesis and clinical improvement of cobalt deficient sheep The doses of vitamin B_{12} required to produce such an effect are large in comparison to the effective therapeutic doses in other species This may indicate an unusually large requirement of vitamin B_{12} by ruminants

Whether cobalt is required by any animal for purposes other than the synthesis of vitamin B_{12} cannot be decided at this time There is little, if any, evidence that it plays any other physiologic role

The hemopoietic activity in man of cobalt containing vitamin B_{12} was reported by the late Dr Randolph West (1948) Its effects are so familiar as to require no further discussion here That cobalt itself is not responsible for the therapeutic effect of vitamin B_{12} in macrocytic anemias has been shown by West and Risner (1949) and by Weissbecker and Maurer (1947) We can conclude, therefore, that a cobalt containing compound has remarkable physiologic activity which is not shared by the trace element itself

Interest in the effects in the human of cobalt administration has been stimulated due to the development of vitamin B_{12} and also to observations by Wintrobe and coworkers (1947) on the effect of cobalt administration in the anemia of infection in rats These workers produced turpentine (sterile) abscesses in rats and observed that a mild depression of hemoglobin resulted In addition, there was a decrease in plasma iron level and an increase in erythrocyte protoporphyrin concentrations in the rats treated with turpentine Daily administration of 0 5 mg of cobaltous chloride intraperitone ally protected the turpentine treated groups from the decrease in hemoglobin and permitted these rats to attain higher than normal hemoglobin levels Cobalt administration did not alter appreciably *the plasma iron levels nor erythrocyte protoporphyrin levels of the* turpentine treated animals It is of considerable interest that growth over a 7 to 11 week period was less than normal for the cobalt treated rats—even those not injected with turpentine This may indicate that cobalt at the level administered was mildly toxic

These clinical effects of the administration of large quantities of cobalt have, to some extent, been confirmed in man Weissbecker and Maurer (1947) reported the production of a true polycythemia in healthy subjects without qualitative or quantitative changes in the leukocyte count following the administration of cobalt salts Intravenous administration of 5 to 10 mg of cobalt per day was followed by temporary toxic symptoms Beginning about the third day, a reticulocytosis and increase in erythrocytes and hemoglobin level occurred These levels rose some 20 to 50 per cent above the initial values and remained there despite giving additional cobalt They gradually returned to normal after discontinuing the salt As in animals, these results are said to have been repressed by admini stration of ascorbic acid

Weissbecker and Maurer reported some favorable effect of cobalt administration (dosage unstated) upon the anemia of blood loss and upon some cases of anemias of infection It was stated that 60 to 80 per cent of the daily intravenously administered cobalt was excreted in the urine within 24 hours The remainder was excreted in the

feces Further, these workers report a serum content of 0 5 to 1 0 mcg per cent of cobalt in healthy individuals

Similar findings in normal subjects were reported by Berk, Burchenal, and Castle (1949) They found that the daily oral administration of 300 mg of cobaltous chloride was followed within a week by a slight reticulocytosis in 17 non anemic subjects In 9 of 10 patients who received the salt over a four week period, there occurred a moderate increase in erythrocytes, hemoglobin, and hematocrit The most striking response was an increase in erythrocyte count from 3 9 to 6 9 million per cu mm over an eleven week period of administration of cobaltous chloride

It is apparent, therefore, that the administration of cobalt salts in what appear to be relatively large amounts has an effect similar in man to that observed in animals, namely, the production of polycythemia

Berk *et al* found that cobalt administration was without effect in 5 patients with idiopathic refractory anemia associated with hypercellular marrow, but had a definitive erythropoietic response in a patient with familial microcytic anemia (Cooley s anemia) In common with a simultaneously reported series of observations (Robinson, James, and Kark, 1949), it was found that patients with so called anemia of infection exhibited some hemopoietic response to the administration of cobalt salts

It would seem important that all workers who have administered pharmacologically active quantities of cobalt to patients or healthy subjects have commented upon the frequent occurrence of anorexia and other alimentary tract symptoms and, in some instances, malaise Two patients in the Boston series experienced precordial pain and exhibited clinical evidence suggestive of myocardial infraction while taking cobalt

Fig 7, kindly loaned me by the late Dr Edgar Jones of the Department of Medicine of Vanderbilt University, depicts the hemopoietic response following cobalt administration to a patient with an anemia associated with chronic infection Both periods of cobalt administration were accompanied by symptoms of nausea, malaise, and chest pain sufficient to warrant interdiction of medication

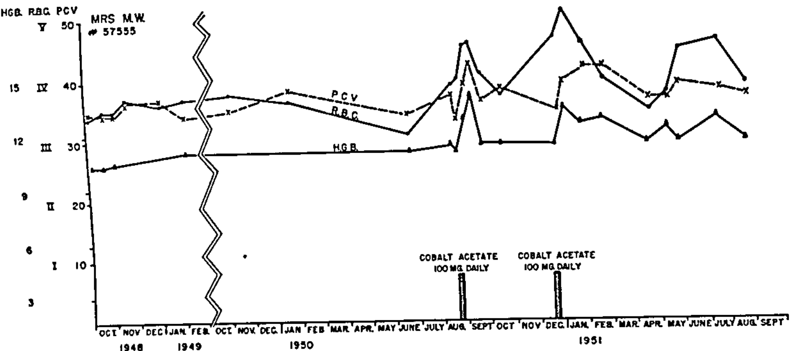


Fig 7 The hemopoietic response to cobalt of a patient with an anemia associated with a chronic infection Both periods of cobalt administration were interdicted because of the appearance of symptoms of toxicity (Patient of Doctor Edgar Jones, data kindly made available by Doctor Jones)

summarize the extensive amount of work carried out on this condition, but suffice it to note that Eden, Hunter, and Green (1945) found a decreased blood copper level of ewes in the region where "swayback" was common in lambs The copper content of the blood increased as the incidence of diseased lambs decreased following therapeutic administration of copper

A number of demyelinating neurologic conditions occur in man and are of unknown etiology Mandelbrote *et al* (1948) have investigated 37 patients suffering from these diseases, including patients with disseminated sclerosis, neuromyelitis optica, and other conditions Copper estimations were made on blood, urine, liver, and brain These studies included the influence of BAL on copper excretion (see McCance and Widdowson, 1946, McDonald, 1946) No evidence was found which would support an hypothesis of an abnormality of copper metabolism in the human conditions studied

Munch Peterson (1950) reported on the copper content of cerebral spinal fluid in children and adults with and without central nervous system lesions Considerable variation in the copper content of the cerebral spinal fluid was noted by this worker, but no consistent pattern was evident which would indicate a relationship between copper and any of the diseases studied

Molybdenum

Because of the interrelationships between copper and molybdenum, this element was discussed at the symposium on copper and the background information on it was well summarized by Comar (1950) and Davis (1950)

Experimental studies in animals have not as yet permitted us to classify molybdenum as an essential trace element (Teresi, Elvehjem, and Hart, 1942) Davis (*loc cit*) has summarized the evidence that in the herbivorous animals small amounts of molybdenum (2 to 25 parts per million) have been associated with an increased requirement for copper and a change in the metabolism of bone Comar, Singer, and Davis (1949) reported studies in rats which support the findings of the dependence of low level molybdenum toxicity upon

the copper intake of the animal They have reported, in keeping with earlier workers, that molybdenum feeding decreases the liver store of copper Grey and Ellis (1950) have also reported that molybdenum retards growth in rats, but they were unable to demonstrate a clear counteracting effect of copper on this toxicity under the conditions of their experiment It is of interest, however, that they found that copper corrected an anemia caused by inclusion of zinc in the diet

No studies of this copper molybdenum relationship seem to have been made in man

Knowledge concerning the toxic effects of molybdenum is summarized by Fairhall *et al* (1945)

Investigation of the possible therapeutic effect of molybdenum in man seems to be limited primarily to studies on a molybdenum iron complex for the treatment of anemia of pregnancy (Dieckmann and coworkers, 1949, 1950, Neary, 1946) Several workers have concluded that significant increases in hemoglobin concentration have occurred following the administration of this compound Unfortunately, the investigations reported to date have not been so designed as to permit the making of valid comparisons between the results of treatment with this preparation and with iron alone in properly classified patients with anemia under identical simultaneous conditions of management Until reports on properly matched samples are available, one cannot decide whether molybdenum does enhance the hematologic response of pregnant women with anemia to the administration of iron Secondly, and importantly, there appear to be no data available upon the metabolic influences of molybdenum administered during pregnancy In view of the animal studies cited above, plus the recognized alterations in serum copper levels which occur during pregnancy, it would seem most important to carry out definitive studies on this relationship between molybdenum and copper in man (Darby, 1950)

ZINC

Excellent summaries of the biological role of zinc have appeared (Hegsted, McKibbin, and Drinker (1945), McClure (1951))

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THE ROLE OF TRACE ELEMENTS IN ENZYME SYSTEMS *

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INTRODUCTION

THE ROLE OF inorganic ions in enzyme catalyzed reactions has, for some years, been of special significance to the biochemists Most workers feel that, like the vitamins, the trace elements or micro nutrients perform their most important function in the nutrition of plants and animals by assuming a catalytic function in enzyme systems That there are other well defined and essential functions for these ions in animal and plant structures is not ignored but is looked upon merely as representing a particular product of an *enzyme catalyzed reaction The task of reviewing the subject of* trace elements and enzymes has been made much easier by several recent publications in this field Lehninger (1950) has discussed in some detail the metalloenzymes primarily from the standpoint of the physical properties of the ions and the structural linkages between metal ions and organic molecules Lardy (1951) has pre sented in some detail, the influence of inorganic ions on phosphory lation reactions and Najjar (1951) has reviewed and presented new evidence concerning the mechanism of action of the micronutrients in enzyme catalysis Rather than attempting to summarize the field of trace elements and enzyme action, therefore, I will attempt to restrict the present discussion to the following points (1) the *influence of inorganic ions on carbohydrate metabolism*, (2) *multiple* metal effects on enzymes and enzyme systems, (3) the metallosub strate concept and (4) the influence of inorganic ions on the growth and metabolic characteristics of organisms as reflected by the altera tion of enzyme patterns

* Contribution number 22 from the McCollum Pratt Institute

INORGANIC IONS AND CARBOHYDRATE METABOLISM

In discussing the role of inorganic ions in carbohydrate metabolism, it is significant that just 20 years ago McCollum and associates first described the spectular syndrome of Mg deficiency in rats and dogs In that same year Lohmann demonstrated the importance of Mg in muscle glycolysis

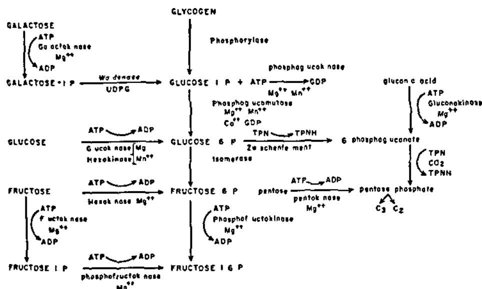


FIG 1 Carbohydrate Metabolism—Site of Action of Inorganic Ions

Since the early observations of McCollum and of Lohmann in 1931 this element has been shown to have played a predominate role in the activity of various enzymes concerned with carbohydrate metabolism The variety of hexokinases which have now been studied in detail all seem to require magnesium ion as an activating metal (See Lardy, 1951) In most instances, maganese will substitute for magnesium, but the activity is usually somewhat lower The crystal line yeast hexokinase in the presence of Mg and adenosine tri phosphate will phosphorylate either glucose, fructose, or mannose (Berger, Slein, Colowick, and Cori) This also appears to be true for the brain hexokinase (Slein, Cori, and Cori) The nature of the reaction is shown in Fig 1 The fact that one sugar will compete with another in this reaction has been taken as evidence that only one

active center is present in the enzyme In contrast to brain and yeast hexokinase, similar enzymes from other sources show a much higher specificity Separate phosphorylating enzymes for each sugar have been found in liver, muscle and microorganisms (see Leloir, 1951) As indicated in Fig 1, specific glucokinase, fructokinase and galacto kinase all require magnesium as the activating metal In the case of fructose and galactose, the phosphate of ATP is transferred to the one position, instead of the 6 position, as is the case with the yeast hexokinase Recently Leloir and associates have been able to show the nature of the conversion of galactose 1 phosphate to glucose-1 phosphate, demonstrating the necessity of a new coenzyme which they isolated and characterized as uridinediphosphate glucose (Caputto *et al*, 1950)

After the separation of glucose and fructose phosphorylating enzymes from muscle and liver by Cori and Slein (1947), con siderable progress has been made in understanding the functional significance of inorganic ions in these reactions Hers (1951) has recently investigated the relationship of ATP, Mg Na, and K in the liver fructokinase reaction In a medium containing a low K concen tration, he found an optimum Mg/ATP ratio of 0 5 By increasing the K concentration to 1M, the enzyme activity was enhanced several fold Under these conditions the optimum Mg/ATP ratio was 1 Na was only slightly stimulatory under these conditions Hers s evidence indicates that the Mg ATP complex is the actual substrate of the fructokinase reaction The affinity of the enzyme for the complex was five times greater in the presence of K than in the presence of Na ions Since higher K concentrations were inhibitory and Mg ATP could competitively reverse this inhibition, Hers suggests that fructokinase has two reactive groups which combine with the metal The active form of the enzyme is obtained when the Mg ATP complex occupies one site and K occupies the other, other permutations are apparently inhibitory The observations of Hers and others concerning double ion requirements of enzymes lends support to the concept of metallo substrates, an idea which has been extensively discussed by Najjar (1951) This concept of metal function will be considered later

Glucose 1 phosphate in the presence of animal phosphorylase, is converted into glycogen, and the work from Cori s laboratory demonstrates that its action consists in a lengthening of the 1, 4 glycosidic chains of the primer polysaccharide No inorganic ion requirements have been reported for this enzyme, but recent evidence by Marks and Shorr (1950) indicates that it may be important in calcification The removal of glycogen from a cartilage slice by amylase action abolishes calcification, however, the deposition of calcium could be restored by the addition of glucose 1 phosphate

The enzyme which transforms glucose 1 phosphate into glucose 6 phosphate, phosphoglucomutase, was discovered by Cori and Cori, and has recently been crystallized by Najjar (1948) The enzyme was known to be activated by magnesium or manganese ions, but quite recently Stickland (1949) has shown that two metal ions are required for maximum activity, magnesium and one metal of a variety tested Chromium could produce single metal activation, but at a greatly reduced rate Since these observations have been made, Leloir and his group have demonstrated that glucose 1, 6 diphosphate is an essential coenzyme for this reaction It would be of considerable interest to reinvestigate the metal ion requirements of this reaction, using the known purified components Paladini, *et al* (1949) have isolated an enzyme from yeast and muscle which, in the presence of ATP, converts glucose 1 phosphate into glucose 1, 6 diphosphate, the coenzyme for phosphoglucomutase This phosphoglucokinase requires magnesium or manganese as an activator

In the presence of a specific isomerase, Lohmann (1933) demonstrated that glucose 6 phosphate is converted into fructose 6-phosphate The latter compound can be phosphorylated by ATP in the presence of phosphofructokinase and magnesium ion (Cori and Slein, 1947, Mang, 1947) The fructose 1,6 diphosphate, in the presence of aldolase, is split into two trioses, phosphoglyceraldehyde and dihydroxyacetone phosphate (Meyerhof and Lohmann, 1934) (see Fig 2) The aldolase of rabbit (Taylor Green and Cori, 1948) and rat muscle (Warburg and Christian, 1943) has been crystallized and that of yeast has been highly purified Warburg and Christian found that the yeast enzyme was inhibited by pyrophos

phate and cysteine, but could be reactivated by adding zinc, cobalt, ferrous or cupric ions Bard and Gunsalus have also reported that iron is essential for the aldolase of *Clostridium perfringens* For the aldolases of peas (Stumpf, 1948) and animals there is no clear evidence of the necessity of metal ions

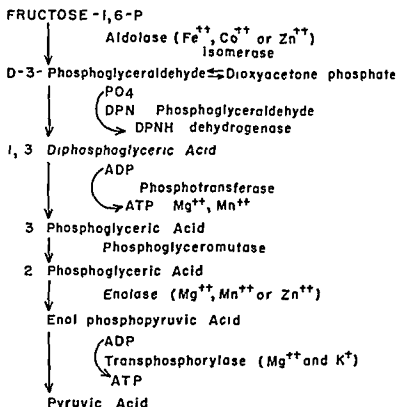


FIG 2 Carbohydrate Metabolism—Site of Action of Inorganic Ions

One of the products of aldolase action, phosphoglyceraldehyde, is oxidized to phosphoglyceric acid in the presence of a specific triosephosphate dehydrogenase, coenzyme I, phosphate and adenosine diphosphate Since no metal ion is required for the action of this dehydrogenase, it will not be discussed in detail The 3 phosphoglyceric acid is converted into 2 phosphoglyceric acid in a reaction catalyzed by phosphoglyceromutase (Meyerhof and Kiessling, 1935) These latter workers also demonstrated the presence of an enzyme (enolase) in the crude preparations of the mutase which was con

cerned in the conversion of 2 phosphoglyceric acid into enol phospho pyruvic acid Later, Warburg and Christian (1942) demonstrated that enolase is a conjugated metal protein The pure protein which they crystallized from yeast juice required either Mg^{++}, Mn^{++}, or Zn^{++} for activity Because of the relatively high dissociation constant for the Mg enzyme complex, most workers feel that this is the natural activator Enolase is strongly inhibited by fluoride, and Warburg and Christian demonstrated that this inhibition depended upon the presence of phosphate In the presence of the latter a magnesium fluorophosphate complex is formed, which displaces the magnesium from the enzyme

The phosphate of enol phosphopyruvate is energy rich, and in the presence of a specific transphosphorylase, can be transferred to the adenylic acid system to form ATP The early work by Kubowitz and Ott (1944) demonstrated a Mg^{+} requirement for this enzyme, however, later investigations by Boyer Lardy and Phillips (1942) demonstrated that K^{+} was likewise required Apparently the irre versible nature of this reaction as earlier observed by Meyerhof, *et al* (1938), using dialyzed muscle extracts was due to the lack of potassium (Lardy and Ziegler 1945) The effect of potassium on the reversibility of this reaction is of particular significance since it would seem to offer an excellent system for studying the mechanism of transphosphorylation in general In addition, it offers another intriguing example of multiple ion activation

Lehninger (1950) has pointed out the possible importance of this transphosphorylase, with regard to potassium accumulation during fermentation in yeast cells and its unequal distribution between mammalian cells and extracellular space Lardy (1951) has also emphasized the importance of complex formation between potassium and some specific cellular constituent as a possible mechanism involved in K^{+} transport and accumulation

A second metal requiring transphosphorylase is that concerned with the transfer of phosphate from 1, 3 diphosphoglyceric acid to ADP Bucher (1947) has recently crystallized this enzyme from yeast and has shown that both Mg^{++} and Mn^{++} are effective metal activators Actually Mn^{++} was much more active than Mg^{++}, but

because of the low concentration of Mn^{++} in the yeast cell, Bucher considers Mg^{++} as the natural activator

This brief survey of the various enzymes concerned in the classical scheme of fermentation and glycolysis will give some idea of the importance of metal ion requirements in this process In addition to enzymes concerned in the Embden Meyerhof scheme, several new metal requiring enzymes have been described for the reactions concerned in the Warburg Lipmann Dickens pathway, in which glu cose is oxidized directly, leading to trioses via pentoses Both Horecker (1951) and Cohen (1951) have recently summarized the evidence for these reaction mechanisms involved in the direct oxida *tion of glucose 6 phosphate Using adaptation techniques, Cohen* has been able to isolate from *E coli*, strain B, several specific kinases for pentoses, as well as gluconic acid The reactions are similar to the hexokinase catalyzed reactions in that a transphosphorylation between ATP and the pentoses is involved magnesium is once again the essential metal activator

It is clear from the summary in Figs 1 and 2 that a divalent metal ion is essential for most of the reactions in the glycolysis or fermenta tive pathways, particularly for those reactions involving intermole cular phosphate transfer Among the divalent ions which are active, magnesium seems to occupy the central role There are a number of enzymes involved in the schemes presented, which do not require a metal for activity, and Lehninger has pointed out that these may be important with regard to investigating the nature of enzyme substrate interaction It is emphasized that in the one group the metal is essential for the attachment of the substrate to the enzyme, while in the second group the attachment of the substrate is made directly to the protein through some non metallic linkage The enzymes of the latter group include phosphorylase, zwischenferment, hexosephosphate isomerase, triosephosphate isomerase, and dehydro genase If the concept of metalosubstrates is correct, the above viewpoints may be slightly altered

In addition to the enzymes concerned in the fermentative pathway leading to pyruvic acid, there are several other metal activated enzymes which are concerned with the metabolism of carbohydrates Some of these enzymes are listed in Table 1 and Fig 3

TABLE 1

THE ROLE OF METALS IN THE ACTIVATION OF ENZYMES OF THE CITRIC ACID CYCLE

Enzyme	Reaction	Metal requirement	Reference
Pyruvic carboxylase	Pyruvic acid → Acetaldehyde + CO_2	Mg^{++} Mn^{++}	Auhagen 32; Green et al 41
Pyruvic oxidase	Pyruvic acid + H_3PO_4 → Acetyl P + CO_2	Mg^{++} Mn^{++}	Lipmann 44; Korkes 51
Oxaloacetic decarboxylase	Oxalacetic acid → Pyruvic acid + CO_2	Mg^{++} CO^{++}, Zn^{++}	Schweet 51; Speck 49; Plaut + Lardy 49
Transacetylase	Acetylphosphate + CoA ⇄ PO_4 + Acetyl CoA	Mg^{++}	Stadtman 50
Isocitric dehydrogenase	Isocitric Acid $\xrightarrow{TPN}$ oxalosuccinic	Mg^{++} Mn^{++}	Adler et al 39
Oxalosuccinic decarboxylase	Oxalosuccinic → ketoglutaric acid + CO_2 + O_2	Mn^{+}	
α ketoglutarate oxidase (plant)	α ketoglutaric acid → succinic acid + CO_2 + HXO	Mg^{++} Mn^{++}	Kornberg et al 48
Succinic dehydrogenase	Succinic acid → fumaric acid + 2H	Ca^{++} Al^{+} OH	Schales et al 50
Malic enzyme	Malate + TPN → Pyruvate + CO_2 + TPNH	Mn^{++} CO	Axelrod et al 42; Horecker et al 39; Salles + Ochoa 50; Conn et al 49

The oxidation of pyruvic acid is known to occur in a variety of tissues, and recent evidence from various sources indicates that several cofactors are involved In the original studies by Lipmann on the oxidation of pyruvic acid by *Bacterium delbrueckii*, the necessity for phosphate was clearly established and acetyl phosphate was isolated as a product of the reaction (see Lipmann, 1949) The activation of acetyl phosphate to function as an acetyl donor, as well as the detailed investigations concerned with is formation, represents one of the more interesting and exciting developments in the field of biochemistry (see Barker 1951) Needless to say, the pyruvate oxidizing system requires an inorganic ion for its normal function, however, the detailed mechanism involved has not been clearly established Although it was earlier reported that the oxidation of pyruvate to acetate required Mg Mn^{++}, or Co^{++} (see

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In the presence of a condensing enzyme from animal tissue, but in the absence of inorganic phosphate and transacetylase, the acteyl group can be transferred to oxaloacetic acid to form citric acid

Recently Schweet, *et al* (1951) have described a pyruvate oxidase preparation from pigeon breast muscle, which leads to acetate formation in the absence of added CoA It would appear that this Mg^{++} requiring system leads to the formation of an acetyl enzyme complex as the normal intermediate It is suggested that the C_2 enzyme complex can now participate in several reactions, depending upon the environmental conditions It can be hydrolyzed to form acetate, or by the addition of suitable enzymes and acceptors be made to acetylate Although such inorganic ions as Mg or Mn^{++} are required for the oxidation of pyruvate, it is clear that much remains to be done before a specific statement can be made concerning the site and mechanism of activity of various metals

The condensing enzyme, which catalyzes the synthesis of citric acid from acetyl CoA and oxaloacetic acid has recently been isolated from pig heart as a crystalline protein (Stern, Shapiro and Ochoa, 1950) The active acetate, in most of these experiments, was obtained from synthetic acetyl phosphate in the presence of CoA, transacetylase, and Mg^{++} ion

The direct demonstration that citric acid is the product of the reaction catalyzed by the condensing enzyme definitely places this tricarboxylic acid as a main intermediate in the Krebs cycle In the presence of the enzyme, aconitase, citric acid rapidly equilibrates with cis aconitic acid and D isocitric acid Although aconitase apparently is not a metal activated enzyme, the recent observations of Dickman and Cloutier (1950) are of interest They have found that the loss of aconitase activity in crude heart extracts exposed to air could be restored by ferrous ion No other metal was effective in this respect

There is some question concerning the metal requirement of isocitric dehydrogenase which was recently purified by Grafflin and Ochoa (1950) The enzyme is, however, not free of oxalosuccinic decarboxylase activity, and, consequently, the product of the reaction studies is α ketoglutarate (Table 1) According to Ochoa, the latter enzyme catalyzed the condensation of Mn^{++} ion with oxalosuccinate,

Lipmann, 1944), it is not entirely clear which are the metal requiring enzymes that are involved From bacterial extracts it has been possible to obtain convincing evidence that acetyl phosphate is not the primary product of pyruvate oxidation, and, in fact, may not be an obligatory intermediate in acetyl transfer from pyruvic acid The

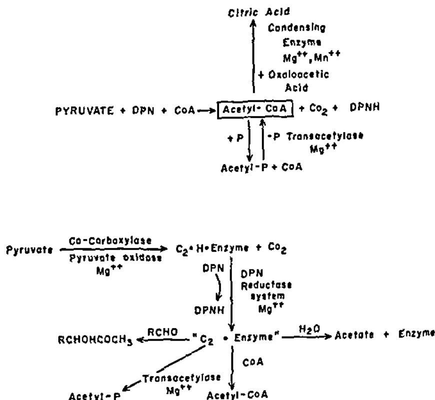


FIG 3 The Importance of Inorganic Ions in the formation of Active Acetate and *Related Processes*

results suggest that acetyl CoA is the primary product of pyruvate oxidation, according to the scheme in Fig 3 According to Korkes (1951) investigation on extracts from *E coli*, which catalyze the dismutation of pyruvate to lactate, acetyl phosphate and CO , in the presence of inorganic phosphate, two enzymes are required for the initial reaction in the formation of acetyl CoA Mg^{++} or Mn^{++} are essential for this reaction In the presence of inorganic phosphate and transacetylase (a Mg^{++} requiring enzyme) the CoA enzyme complex undergoes phosphorolysis to acetyl phosphate and CoA

Other Metal Activated Enzymes

In addition to those enzymes listed in Figs 1, 2, 3 and Table 1 and discussed in connection with carbohydrate metabolism, there are listed in Table 2 other metal activated enzymes which are of interest

TABLE 2

Metal Activation in Transphosphorylation Reaction

Enzyme	Reaction	Metal	Reference
Myokinase	2 ADP ⇆ AMP + ATP	Mg^{++}	Colowick and Kalckar 43
Adenosinekinase	Adenosine + ATP → ADP + AMP	Mg^{++}, Mn^{++}	Caputto et al 51
Creatine phosphopherase	Creatine + ATP ⇆ Creatine Phos + ADP	Mg^{++}	Lehman 35
Arginine phosphopherase	Arginine + ATP ⇆ Arginine Phos + ADP	Ca^{++} Mn^{++} Mg	Szorenyii et al 49
Flavokinase	Riboflavin + ATP → riboflavin Phos + ADP	Mg^{++}	Kearney and Englard 51
DPN phosphopherase	DPN + ATP → TPN + ADP	Mg^{++}, Mn^{++}	Kornberg 51
Glutamic phosphokinase	Phosphorylation of glutamic acid	Mg^{++}	Speck 47
Firefly luciferase	ATP + LH_2 + O_2 → light + L	Mg^{++} Mn^{++} CO^{++} Fe^{++}, Ni^{++}	McElroy + Strehler 49
ATPase	ATP + H_2O → AMP + $2PO_4$	Mg^{++} or Ca^{++}	Numerous
Yeast apyrase		Mn^{++}	Meyerhof 45
Potato apyrase		Ca^{++}	Kalckar 44
Nicotinamide Methyl kinase	Methylation of Nicotinamide by Methionine	Mg^{++}	Cantoni 50

No attempt has been made to insure that the list is complete In addition, most of these examples have been adequately discussed in recent reviews

The metal requiring enzymes listed in Table 2 catalyze reactions concerned in the use or transfer of high energy phosphate groups from ATP Lardy (1951) has recently discussed in some detail the function of metal ions in these reactions Most of these reactions

the product of isocitric acid oxidation, to form a complex which decomposes during decarboxylation The changes which occur are similar to those described by Kornberg, Mehler, and Ochoa (1948) for oxaloacetate decarboxylation

Ketoglutarate oxidation leads to succinic acid, and recently Schales, *et al* (1950) have described a Mg^{++} or Mn^{++} requirement for such an oxidase isolated from higher plants Recently Kaufman (1951) has implicated CoA in the oxidation of α ketoglutarate, and the results indicate the intermediary formation of succinyl CoA, which, when hydrolyzed, yields succinic acid and CoA In the presence of phosphate and ADP, however, ATP is generated Several enzyme preparations of succinic dehydrogenase have been described, and recently Scott (1950) has succeeded in separating it from cytochrome *c* reductase activity None of the preparations of succinic dehydro genase described requires a metal for activity However, Axelrod, *et al* (1942) reported its activation by calcium ion, but this latter effect is apparently due to an inhibition of the formation of oxalo acetic acid, which in turn inhibits succinic dehydrogenase The product of succinic acid oxidation, fumaric acid, is rapidly converted into malic acid in the presence of a non metal requiring enzyme, fumarase

Several metal requiring enzymes concerned in the metabolism of oxaloacetic and malic acid have been described Ochoa and associates have clearly demonstrated that the malic enzyme catalyzes the oxidative decarboxylation of malate to pyruvate and Co without free oxaloacetic acid being an intermediate Mn^{++} ion is required for this reaction In addition, Ochoa and Salles (1950) have recently described the purification of oxaloacetic acid decarboxylase which likewise requires Mn^{++} ion, and is very closely associated with the malic enzyme Conn, *et al* (1949) have described a malic enzyme obtained from higher plants whose Mn^{++} requirement could be replaced by cobalt

Where Mg^{+} appeared to play a predominate role in the glycolytic cycle, it appears that Mn^{++} is the predominate ion in the citric acid cycle and this effect would appear to be closely related to the impor tant oxidative and non oxidative decarboxylation steps which occur during the metabolism of di and tricarboxylic acids

Other Metal Activated Enzymes

In addition to those enzymes listed in Figs 1, 2, 3 and Table 1 and discussed in connection with carbohydrate metabolism, there are listed in Table 2 other metal activated enzymes which are of interest

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have not been studied in great detail insofar as their metal ion requirements are concerned Probably the ATPase are the best known with respect to metal ion activation, however, Mommaerts (1951) has recently demonstrated that much remains to be investigated with respect to ATPase activity and metal ion requirements in the actomyosin complex The finding of Kielley and Meyerhof (1948) that a second ATPase is present in muscle, which is activated by Mg instead of calcium, also complicates the picture insofar as ATP utilization is concerned The present evidence would seem to indicate that the Ca activated ATPase is the one more closely associated with the contraction process, and that the Mg/Ca ratio is important in channeling the direction of energy flow The dependence of the physical state and other properties of the actomyosin system on the relative concentration of these ions has been recently summarized by Mommaerts (1951)

The requirements for inorganic ions in the bioluminescent reaction have been studied in some detail, and the results are of interest with regard to ATP utilization (McElroy and Strehler, 1949) With an optimum concentration of magnesium ion, there is an initial bright flash of light when ATP is first added to the purified enzyme obtained from fireflies The light intensity, however, rapidly decreases to a low residual level, due to the removal of ATP in some unknown reaction A variety of experiments indicates that the ATP is still present in the reaction mixture, but is unavailable for maximum light production The rate of immobilization of this ATP depends upon the inorganic ion employed in the luminescent reaction The rate of decrease of light intensity, i e, removal of ATP, is fastest with Mg^{++}, Mn^{++} and Co^{++} are slightly less effective while Fe^{++} and Ni^{++} are only 15 to 20 per cent as effective as Mg^{++} The initial light intensity is correspondingly affected by these different ions The results of the metal ion activation of the utilization of ATP in the luminescent reaction is an unusual example in that ions other than Mg, Mn and Ca are effective In most reactions involving ATP, it is usually found that the latter three ions are the only ones which are effective The fact that others have been found effective in the light reaction may merely represent the greater facility with which one can measure

the reaction and that similar studies on other enzyme systems will show the same relationship In addition to the enzyme reactions listed in Table 2, there are many other less well defined systems which utilize ATP and require a metal ion for activity The system concerned in the synthesis of glutathione from glutamic acid, cysteine, and glycine has been shown by Johnston and Block (1951) to require Mg and recently Grisolia and Cohen (1951) have shown that the

TABLE 3

NON SPECIFIC METAL ACTIVATION OF ENZYMES

Enzyme	Reaction	Metal Req	Ref
Yeast phosphatase	Glycerophosphate → glycerol + PO_4	Mg^{++} Mn^{++} Co^{++} Fe Ni^{++}	Massart and Vandendriessche 40
Acid and alkaline phosphatases	Numerous substrates	Mg Mn and others	See Tauber 49
Arginase	Arginine + H_2O → ornithine + urea	Mn Co^{++} Ni and Fe	Numerous
Lecithinase	Lecithin splitting at phosphorylcholine linkage	Ca Mg Co Zn Mn^{++}	Zamecnik et al 47
Cysteine desulfhydrase	Cysteine → H_2S + NH_3 + $CH_3COCOOH$	Zn Mg Mn	Binkley 43
Pectinpolygalacturonase	Hydrolysis of pectic polyuronides	Ca Na Al	Pallman et al 46
Desoxyribonuclease	Depolymerization	Mg Mn Co Fe^{++}	Miyaji and Greenstein 51

purified enzyme preparation which condenses carbamylglutamate with CO and NH_3 requires ATP and Mg Thus one must conclude with Lardy (1951) that it would be a sound practice to include magnesium in reaction mixtures where new ATP requiring reactions were being sought

Additional metal requiring enzymes are presented in Tables 3 and 4 Those in Table 4 show, in most cases a fairly specific requirement for a metal in contrast to those which have been discussed previously The inorganic pyrophosphatases are extremely specific for magnesium and Bailey and Webb have shown that most other ions which normally can replace Mg in enzyme reactions are quite inhibitory The iron and copper enzymes have been extensively discussed by recent reviewers, and they will not be covered in the

present paper Mention should be made, however, of the recent reports on ascorbic acid oxidase

TABLE 4

SPECIFIC METAL ACTIVATION OF VARIOUS ENZYMES

Enzyme	Reaction	Metal Req	Ref
Carbonic Anhydrase	$CO_2 + H_2O \leftrightarrows H_2CO_3$	Zn	Keilin and Mann 40
Inorganic Pyrophos phatase	Pyrophosphate + $H_2O \rightarrow PO_4$	Mg	Bailey and Webb 44
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$	Fe	See Granick and Gilder 47 *Lemberg and Legge* 49
Peroxidase (Several)	H_2O_2 oxidation of aromatic amines	Fe	Lemberg and Legge 49
Cytochromes	Electron transport	Fe	Lemberg and Legge 49
Tyrosinase	Tyrosine + 1/2 $O_2 \rightarrow$ Hallo chrome	Cu	See Nelson and Dawson 44
Laccase	Phenols $\rightarrow$ ortho and para quinones	Cu	Dawson 50
Ascorbic acid oxidase	Ascorbic acid $\rightarrow$ dehydro ascorbic A	Cu	Dawson 50
Prolidase	Glycylproline $\rightarrow$ proline	Mn	Fruton 46
Dehydropeptidase	Glycyldehydrophenylanine $\rightarrow$ glycine, + NH_3 + phenyl pyruvic A	Zn	Yudkin and Fruton 47
Carboxypeptidase	Chloroacetyl tryosine $\rightarrow$ tyrosine	Mg	Smith and Hanson 49
Glycylglycine Dipeptidase	Glycylglycine $\rightarrow$ glycine	Zn	Linderstrom Lang 34
Glycylglycine Dipeptidase	Glycylglycine $\rightarrow$ glycine	Co (Mn)	Berger and Johnson 39
Carnosinease	Carnosine $\rightarrow$ alanine + histidine	Zn Mn	Hanson and Smith 49
Leucine Aminopeptidase	Leucylglycine $\rightarrow$ leucine	Mg Mn	Berger and Johnson 39
Tripeptidase	Leucyldiglycine $\rightarrow$ amino acids	Zn^{++} (Co^{++}) and a halide ion	Johnson 41

Dawson (1950) and his associates have demonstrated that the copper in highly purified ascorbic acid oxidase is not free to exchange with isotopic copper in solution, as long as the enzyme is in a resting condition In the presence of the substrate and oxygen, however, rapid exchange occurs In the latter case the exchange was

accompanied by a loss in oxidase activity In experiments with ion exchange columns, however, Dawson could demonstrate that the inactivation of the enzyme was not due to a rupture of the Cu protein complex, either in the reduced or oxidized state Additional experiments indicated that none of the products or reactants were responsible for the observed exchange during catalytic activity Dawson proposes that the copper is divalent initially, shuttles reversibly between the divalent and monovalent states during catalysis, and that the exchange of the copper occurs only when the enzyme copper is monovalent The reasons for the last suggestion is based upon the relative bond strengths of the cupric and cuprous states Overall, the experiments suggest the formation of ternary complex of oxygen, enzyme, and substrate

Recently Miyaji and Greenstein have shown that in addition to Mg^{++} and Mn^{++} ion activation of desoxyribonuclease Co^{++}, Fe^{++}, Ca^{++}, Ni^{++}, Cd^{++} and Zn^{++} are also effective Cobalt and iron were about as effective as Mg^{++} The conditions under which the optimal activation occurred, however, varied with the ion The optimum pH for the activation with Mg^{++}, Co and Ca was 6 5, that for iron was 5 7, while two optima for Mn were observed (6 8 and 8 0) The point of physiological interest is that under one condition Mg^{++} is effective, and iron is not, while the reverse can also be true These results are similar to those which were earlier described by Hellerman and Stock (1938), in which they observed a marked difference in the pH activity curves of arginase when different metal ions were used as activators (Mn^{++}, Co and Ni^{+}) At that time, Hellerman postulated the formation of a metal enzyme complex and that the pH activity curves represented the ability of this complex to form coordinate linkages with the substrate molecule Support of these ideas was obtained from pH titration curves in the presence and absence of these ions Smith (1949) has recently suggested a similar mechanism for the metal ion activation of peptidases He pictures the metal forming an asymmetrical chelate complex with protein and the substrate Presumably in this state an electronic deformation occurs at the sensitive peptide linkage, rendering it susceptible to acid or base catalysis at neutral pH The details of this proposed

mechanism of metal activation of peptidases has been recently reviewed by Smith (1951) A few of the peptidases and their metal requirements are listed in Table 4

Metallosubstrates

The concept of a metal chelate complex between substrate and enzyme, as proposed by Hellerman and by Smith, represents, as Najjar points out, an evolution from the parent concept of metal catalysis of purely chemical reactions Recent work on a variety of enzymes offers excellent examples the decarboxylation of oxaloacetic acid by Mn, Cu catalysis of ascorbic acid oxidation, hydrogen per oxide decomposition by iron salts, and others That these examples represent only certain types of reactions is evident from the data which have been presented It is clear that there are many enzyme catalyzed reactions with metal requirements where no catalytic activity is observed, unless the protein is present, and, in addition, there are those enzymes which apparently do not require metal ions for their catalytic effect In the latter cases, it seems clear that the substrate must combine with some reactive group in the protein, in order to obtain the catalytic effects Apparently the metal requiring enzyme either does not have these groups, or the substrate is not reactive to them Thus, it seems logical to propose that the metal functions in a way so as to bring the substrate and the enzyme into an appropriate combination, so that catalysis is brought about Whether the concepts of Hellerman and Smith will satisfy all con ditions is doubtful, but certainly it offers a reasonable approach to a very difficult problem Recently Najjar has proposed an alternative interpretation of metal catalysis, which is worthy of further con sideration Instead of regarding the function of a metal as being that of labilizing a bond he suggests that it may be required simply to form the true substrate of an enzyme Najjar argues it is possible that the metal of the substrate complex forms one of the anchoring points on the surface of the enzyme, thus allowing subsequent attachments between the substrate proper and the protein In other words, the coordinated chelate complex between the metal and

substrate forms the true substrate for the enzyme This viewpoint leads one to conclude that labilization of specific bonds need not be a metal function, and, of course, this in keeping with the facts as pointed out previously In addition, the hypothesis is quite plausible with regard to the action of more than one metal in an enzyme catalyzed reaction Strict configurational requirement would not be necessary, provided the chelates were similar enough so that the orientation of the polar groups of the substrate were within attractive range of the recipient groups on the enzyme surface

Najjar s viewpoints would seem to offer a possible explanation of the multiple metal requirement of certain enzymes which have already been discussed The results of Hers which is striking support for Najjar s concept, indicate that a Mg ATP complex is one of the true substrates in the fructokinase reaction Other metallosubstrate complexes of a number of organic acids and amino acids have been described, and the results obtained in enzyme studies are in no way against the metallosubstrate concept The theory developed by Hellerman and Smith would seem most appropriate for those metal enzyme catalyzed reactions in which there is a high degree of metal specificity, such as pyrophosphatases (Mg), carbonic anhydrase (Zn), and ascorbic acid oxidase (Cu) Needless to say, no proof exists as yet concerning the function of metals in enzyme reactions The theories proposed by Hellerman, Smith, and Najjar form a working framework which will undoubtedly be a fruitful guide for future research

Trace Elements in Growth and Function

Arnon (1950) and Hewitt (1951) have recently discussed the general problem of essentiality of micronutrient in plant growth and function Both authors have emphasized the difficulty of arriving at some decision concerning essentiality as long as multiple activation of enzyme systems is possible The demonstration that certain enzymes or even metabolic systems can be activated by apparently nonessential elements such as nickel would appear to be an obstacle difficult to overcome insofar as essentiality is concerned However, I think that it is clear that it is only necessary to show once a vital

and irreplaceable function of a metal to establish its essentiality The demonstration, for example, that Mg is an essential component of the chlorophyll molecule is adequate evidence that this element cannot be eliminated from the diet irrespective of its possible replace ment in other systems The question then arises how many metals are there in which we can show a specific, non replaceable function in plant and animal metabolism Among the microelements, iron, copper, zinc and manganese are considered essential for both plant and animal development, while boron and molybdenum are required by plants only, and cobalt and iodine are necessary for animals Among these micronutrients there is known at least one specific, non replaceable function in an enzyme system for all the metals except boron and manganese Iron and copper are known to be essential for the function of a variety of oxidases in both plants and animals No other metal can replace them in this respect Zinc is an essential part of the structure of carbonic anhydrase and more recent studies suggest its importance in other enzyme systems Although the enzyme systems in which molybdenum participates have not been obtained in cell free preparations, several authors have shown conclusively that it is essential for nitrogen fixation and nitrate utilization in plants Additional evidence has also been pre sented by Hewitt and others which suggests its importance in amino acid metabolism Cobalt is an essential element in the vitamin B_{12} and considerable evidence is accumulating to indicate that, along with folic acid, it is important in the metabolism of sulfur containing amino acids Chow and associates have shown that the blood of *pernicious anemic patients is quite low in sulfhydryl groups* and in some cases the administration of B_{12} produces a rapid rise to normal blood levels This result has been confirmed with experimental animals on a diet low in B_{12} Iodine has received more attention than probably any other trace element in nutrition The early detec tion of iodine in the thyroid gland by Baumann and the subsequent isolation of the iodine containing active hormone, thyroxin, by Kendall, established beyond doubt the importance of this element in the diet Other functions of iodine are not known but it appears

certain that metabolic processes are affected by this element other than through the action of thyroxin or one of its derivatives

Let us return now to the multiple metal effect Although we can describe a specific function for most of the metals it is evident as long as there are essential enzyme systems which can be activated by a variety of elements we can expect nonessential metals to have a significant effect on the metabolism of plants and animals Some of the most striking examples are from the area of plant animal relationship In the reclamation of certain soils in Australia it was necessary to apply large quantities of molybdenum and phosphorus to obtain adequate and healthy growth of plants The consumption by cattle of this high molybdenum containing forage resulted, however, in aggravated symptoms of copper deficiency Although the reasons for this molybdenum copper relationship are not clear, it is evident that a deficiency disease may not merely reflect a low level of a dietary essential but may also indicate an excess of one or more minerals which interfere with the normal function of an essential constituent

There are many examples of multiple metal effects on known enzyme systems and a few of these were previously discussed As judged by the optimum concentration of an element for enzyme activity as well as the ratio of stimulatory and inhibitory metals present, it is evident that under physiological conditions some systems may be working at a maximum rate whereas others may be operating at less than 1 per cent of maximum efficiency Obviously there must be some balance in the soil, in the plant and in the animals which leads to a normal, healthy individual However, the difficulty of determining what is the "normal" activator of a metabolic system, and factors which will influence the rate of reaction *in vivo*, is exemplified by the experiments of Barrenscheen and Valyi Nagy on transmethylation in liver homogenates In the methylation of guanidoacetic acid by the amino acid, methionine, Mg is an essential ion However, when guanidoacetic acid is supplied as the only substrate, the methyl donor being supplied by an endogenous source in the tissue extract, Mn, Co and Fe were effective activators of the transmethylase When methionine was added, as the methyl source, Mn had no effect,

the enzyme accelerates the destruction of a metabolite known to be essential in many biological reactions There may be relationships of this type to the functioning of certain processes in the human body but unfortunately very little research has been conducted along this line Such observations, however, on altered enzyme activity may be of value in the early diagnosis of animal and plant deficiencies which are not apparent from growth studies and in addition provide us with information as to how plant and animal enzymes can be altered to give an increased yield of a desired product whether it be concerned with animal health, the aroma of tobacco, penicillin production or nitrogen fixation in the soil

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willing to accept that level of activation as preliminary evidence for a requirement of an inorganic element for enzyme activity I think that the same statement could be made with respect to other types of activation, for example, the supposed requirements of potassium for the transfer of phosphate from enol phosphopyruvic acid Potassium may not be absolutely required for this reaction, but it is definite that potassium has an accelerating influence on the reaction, and I think undoubtedly that this will have physiological effects on the organism I don't see the arguments that this in any way invalidates the hypothesis proposed by Hellerman and Smith I think in fact that this is some of the best evidence for that hypothesis It's true that we don't know how metals work in enzyme systems but I think those two pieces of evidence as presented by Hellerman and Smith and the other concept which was recently discussed by Najjar is the best working hypothesis that we have at the present time They are definitely not proven as yet

DR VALLEE I only meant to point out the existence of the dichotomy in this field The reference to these papers was not meant to imply the rejection of any hypothesis but merely to emphasize that they constitute negative evidence for the presently existing theories

MR PRATT Are there any other comments or questions?

DR TOURTELLOTTE Since it has been Dr McCollum's eternal thesis that we should look to our common foods for our normal nutrition it immediately takes us back to the soil and I think that since we have with us one of our country's top soil experts—that's not meant to be a pun—but we have Dr Bear from Rutgers who has been doing remarkable things in putting these trace elements into the soil for improving and farming an ocean bottom so to speak In other words, it rests on him to keep New Jersey still the "Garden State" I think that he might have some ideas here

MR PRATT Dr Bear, we'd be very glad to hear from you

DR BEAR I have a lot of ideas but I hesitate to express them I am impressed by one thing I have been reading more recently Sir Albert Howard's book on the Indore system of making compost He has a lot of followers in this country He believes that you can't produce a good quality of food without using compost and that the compost must contain human and animal excrement as well as plant waste From time to time I have assigned my students to review that book Each time a student is assigned to the job of reviewing it he takes a negative attitude on it and tends to try to refute what Sir Albert has to say I'm impressed today that Dr Darby was taking what might be called a negative attitude on minor elements, that is he has to be shown I think we'd get a good bit further with a positive approach which is to the effect that they are essential, and consider how this essentiality comes to be

I deal with soil and plants and I am so impressed by the essentiality of a number of these minerals in terms of plant yield and quality that I find it difficult to believe that similar important values are not present in animals as well In fact, I think the presentation which we have just had indicates that they are valuable both to plants and animals although these two groups require somewhat different sets of elements In other words we know that boron and molybdenum are required by plants and cobalt and iodine by animals, whereas the other three mentioned—manganese, copper, and zinc—are required by both plants and animals

I should like to raise a question that has been bothering me for a long time As I recall it, life itself originated in the ocean and, in due time, became differentiated into plants or animals as the case may be We crawled out of the ocean and started on our way This ocean from which we came is an organic salt mixture that is something to contemplate All the known elements that are present in the soil are carried into the sea Presumably they are being at least tolerated by the organisms that live in the sea One of the things that disturbs me a great deal is the modern tendency toward purification of salt That is, we take everything out of sea salt except sodium chloride, add a trace of potassium iodide and call that a nutritional salt What I should like to know is as to who decided that that ought to be done There s a lot of evidence to make one believe that s a mistake Someone this afternoon mentioned that a great many people use sea salt instead of sodium chloride I am very much interested in that point Is there any objection to using sea salt or is there anything that can be said in favor of pure sodium chloride?

MR PRATT Can anyone offer Dr Bear an answer there?

DR DARBY I think there are two aspects to this problem of essentiality and deficiency diseases and I should like to declaim to the extremists in which I would classify the book to which you referred One is whether an element is essential Now let s take as a specific example the case of zinc I think we would be going a long way to deny that zinc is probably essential for man It is present in an important enzyme system in the body It would be surprising if evidence appeared that zinc was not essential for man On the other hand, in reference to this particular evidence I know of no clear cut demonstration that zinc deficiency has ever been produced in man nor has it been found spontaneously occurring Now I think we sometimes fail to distinguish between those two questions—the essentiality of not only trace elements but with vitamins and amino acids and the occurrence of deficiency manifestations in man Now it happens that man isn t a plant nor is he a raving animal He gets his food from many many places and in this country at least with modern methods of food production and transportation of food and our most remarkable American grocery store, it s quite difficult indeed

DR McELROY I'd like to ask Dr Darby what criteria does he use in animal experimentation to decide what are the essentialities when he started out to demonstrate such a phenomena Would you think it was necessary to demonstrate an effect on growth or some pathological effect in the body when you came to a conclusion that this element was essential?

DR DARBY I don't think there is any single criterion which one can use to demonstrate the essentiality of any nutrient I think if you wish to investigate whether or not a nutrient is essential then you must, so far as you can determine, remove that nutrient as completely as possible from the diet and study what happens to the animal and that means as many things as you can observe

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